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14. ABSTRACT

An insiduously terrifying aspect of breast cancer is its propensity to recur in metastatic sites even over a decade after all evidence of cancer has passed. It is obvious that these cells had escaped very early from the primary tumor as this occurs even in small, node-negative and in situ primary lesions, and that these micrometastases survival chemotherapeutic regimens that shrink and extirpate the primary carcinomas. Thus this mortal turn of events leads to three key questions – how do the cells escape early?, how do they survive over extended periods?, and what causes these dormant lesions to become aggressive at these late dates? The area of metastatic dissemination of primary cells has received a great level of inspection with an understanding of underlying molecular mechanisms, even if we do not yet have therapies. While chemotherapy survival in ectopic sites has been studies more recently, this is usually done in the context of a growing lesion. The truly under-appreciated and under-studied aspect is the last, that of re-emergence from dormancy. *Understanding what triggers dormant breast cancer cells to emerge and form frank and mortal metastases would allow the development not only of rationale therapeutics but of prevention and possibly lifestyle avoidance.* Herein, these issues are addressed using a novel organotypic bioreactor in which tumor cells can be followed for weeks to months, the process of seeding, dormancy and emergence can be followed.

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Escape from Tumor Cell Dormancy

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ESCAPE FROM TUMOR CELL DORMANCY
An Organotypic Liver System to Study Tumor Cell Dormancy
Alan Wells and Donna Stolz (UPitt), Linda Griffith (MIT)

INTRODUCTION: An insiduously terrifying aspect of breast cancer is its propensity to recur in metastatic sites even over a decade after all evidence of cancer has passed. It is obvious that these cells had escaped very early from the primary tumor as this occurs even in small, node-negative and in situ primary lesions, and that these micrometastases survival chemotherapeutic regimens that shrink and extirpate the primary carcinomas. Thus this mortal turn of events leads to three key questions – how do the cells escape early?, how do they survive over extended periods?, and what causes these dormant lesions to become aggressive at these late dates? The area of metastatic dissemination of primary cells has received a great level of inspection with an understanding of underlying molecular mechanisms, even if we do not yet have therapies. While chemotherapy survival in ectopic sites has been studies more recently, this is usually done in the context of a growing lesion. The truly under-appreciated and under-studied aspect is the last, that of re-emergence from dormancy. *Understanding what triggers dormant breast cancer cells to emerge and form frank and mortal metastases would allow the development not only of rationale therapeutics but of prevention and possibly lifestyle avoidance.*

The dearth of experimental insights into dormancy and the transition that heralds metastatic emergenceis due mainly to the lack of tractable experimental systems with which to probe this critical question. We proposed to use a novel ex vivo liver bioreactor to study this question. *We adapted this liver bioreactor to the study of metastatic competency in our original BCRP-funded work.* Metastases to the liver is one of the three main sites of metastasis and a major site for metastatic emergence after many years. We proposed that dormancy and emergence from it are linked together. Our model of metastatic seeding posited that the disseminated cancer cells undergo a reversion of the initial EMT, to re-establish E-cadherin-based connections in the distant soft organs. These E-cadherin adhesions would not only provide survival signals but also limit proliferation – the definition of dormancy. We proposed to take the next step and *hypothesized that the microenvironment surrounding the dormant micrometastasis is induced to produce growth factors and/or cytokines that downregulate E-cadherin, relieving the breast cancer cells from suppression.*

BODY: The accepted Statement of Work (Table 1) described a series of tasks to accomplish the two Objectives. We have tackled these Tasks in the order of greatest yield so that work in areas can progress as systems are being optimized in others. The main efforts during the first year of this two-year project have been focused on the establishing the system to be tested during the second year.

Table 1. Statement of Work
Work to be performed at University of Pittsburgh (Wells and Stolz Laboratories):
Objective 1:
1. isolate human hepatocytes and endothelial cells (months 1-24)
2. optimize protocols for isolation of human stellate and Kupffer cells (months 1-6)
3. isolate human stellate and Kupffer cells (months 7-24)
3. seed bioreactors with cells (months 1-24)
4. label tumor cells for fluorescence (months 1-6)
5. label tumor cells for mass reporting (months 3-9)

Objective 2:
1. generate liver organ bioreactors for tumor cell seeding (months 3-24)
2. seed organotypic liver bioreactors with tumor cells (months 3-24)

3. select and introduce inflammatory factors (months 9-24)
4. select and introduce stimuli to initiate 'inflammation' in situ (months 15-24)

Work to be performed at MIT (Griffith Laboratory):
Objective 1:
1. design bioreactor scaffolds (months 1-12)
2. optimize new high throughput bioreactor (months 1-12)
3. produce bioreactor scaffolds (months 1-24)

Objective 2:
1. produce bioreactors and scaffolds for utilization (months 1-24)
2. optimize the stiffness of the scaffolds (months 6-24)
3. optimize bioreactor sampling and input ports (months 9-24)

Work at University of Pittsburgh
Objective 1: The five tasks are proceeded apace. We optimized the protocols for isolating the hepatocytes and non-parenchymal cells. The hepatocytes are routinely obtained mainly from human livers discarded as part of resections for colorectal metastases. We also have a protocol optimized for rat hepatocytes.

For the non-parenchymal cells, we have a protocol optimized to collect the entire fraction. This is good for both human and rat fractions. Separating the components is a current focus with an optimized protocol for both the large vessel and sinusoidal endothelial cells. The protocols for the Kupffer and stellate cells will be adapted to the tumor metastasis bioreactor from existing protocols for organotypic bioreactors.

The various breast carcinoma cells are were labeled by chemically and genetically. Cell tracker had been used to stain the cells so we can visualize membranes and shapes rapidly with minimal manipulations. This is useful for primary cells in that it is fast and does not require passaging. This will last for 7 to 14 days depending on the proliferation rate of the cells (as the label both leaches and gets distributed between daughter cells). For the cell lines, we have expressed various fluorescent proteins (mainly GFP and RFP). This labelling lasts for weeks and is maintained even after cell division as it derives from a CMV promoter-driven transcription. However, as the (G/R)FP requires cell transfection, selection and flow sorting, it is not appropriate for primary cell isolates with limited expansion potentials and the need for polyclonal representation.

Thus, the tasks for Objective 1 have been largely completed.
Objective 2: The tasks for Objective 2 are still in progress.

The standard bioreactor with stiff-matrix scaffolds was used to seed tumor cells and test for responsiveness to chemotherapeutic agents (see Chao et al, Clinical and Experimental Metastasis). Even in the partially-activated environment of this stiff-sided bioreactor (where cells along the stiff wall are subject to activation), the presence of the microenvironment confers a differentiation-dependent partial resistance to a broad spectrum of anti-cancer agents. This differentiation state effect is a partial reversion towards a more epithelial phenotype (see Chao et al, Cancer Microenvironment); while E-cadherin is upregulated and makes cell-cell connections, vimentin and other mesenchymal markers remain expressed.

During the second year, we also examined the role of inflammatory cytokines on providing 'an opening' for metastatic seeding. We found that these cytokines, and also autocrine growth factors produced by tumor cells, caused the hepatocytes to undergo a transient dedifferentiation. This broke some of the cell-cell adhesions (E-cadherin downregulation) enabling the carcinoma cells to intercalate between these normal cells and assume a physiological space in the parenchyma. Interestingly, despite the seeming dedifferentiation of the parenchyma, the tumor cells assumed a more differentiated state within a week of seeding among these cells. Heat shock

of the liver cells also accomplished the same 'accommodation' of the carcinoma cells. This work is nearing preparation for submission.

Additionally, as we were optimizing the bioreactor, we investigated the effect of isolated nonparenchymal cells on carcinoma cell phenotype. Unexpectedly, the endothelial cells promoted carcinoma cell growth and survival; the carcinoma cells were able to survive and even undergo repeated mitoses in the absence of serum (without the endothelial cells, the cells underwent apoptosis). However, the carcinoma cells remained in the mesenchymal-like state. Thus, we are pursuing this finding by challenging the postulate the activated endothelial cells promote carcinoma EMT, and may be a trigger for outgrowth.

All of these findings led us to ask whether the dormant phenotype could be accounted by balance proliferation and death of cycling carcinoma cells, rather than our proposed quiescence. The implications for therapy during dormancy are obvious, in that cycling cells might be susceptible to chemotherapeutic agents that nonspecifically kill cycling cells. A simple, unbiased approach was used. We used a Markov chain Monte Carlo simulation to define survival boundaries for nests of cells to remain subclinical (<~0.2gm, or 1 million cells) over an extended period (1218 cycles representing 5-10 years at 1.5-3 days per cycle). Even using stochastic probabilities and starting from 2 cells to 4000 cells, subclinical proliferation was noted only across a singular percentage of survival (49.7-50.8%). These findings, recently submitted for publication (Taylor et al, submitted), strongly implicate quiescence as being a major player in dormancy.

Work at MIT

The bioreactor format has traditionally used thin (0.2 mm thick) wafer-like scaffolds crafted from silicon, polycarbonate, or polystyrene by etching or drilling an array through-holes. The scaffold is then placed into the bioreactor on top of a 5um pore-size filter, thus creating an array of tiny wells into which cells are seeded. Cells attach to the walls of the through-holes but not to the filter, allowing the flow of medium through the cell mass to be reversed following the initial attachment period. Creating a soft gel scaffold format that is functional in all aspects (sturdy, stable, and physiologically relevant) is a multi-step process involving integration of desirable biological properties with desirable fabrication and mechanical properties. From the biology standpoint, cells must initially attach to the gel material in a biologically-relevant manner (i.e., one that does not promote excessive spreading or lack of cell-cell cohesion) and maintain attachment with minimal or modest remodeling of the gel, and the gel cannot globally degrade over the time course of the experiments although local remodeling may be desirable. From the fabrication and mechanical standpoint, a major challenge is the relative fragility of gels compared to stiff substrates, which affects both the handling properties as well as the sturdiness under flow forces. A preferred approach is thus to create gels that are either covalently bonded to the filter material surface or that penetrate into the filter during gelation and form physical links around filter structures, and to then support these filter-gel constructs with a frame that fits into the bioreactor.

We have created free-standing microarrays of PEG-fibrinogen gels that facilitate formation and maintenance of 3D hepatocellular tissue structures (Williams et al, 2011) and demonstrated maintenance of liver tissue function in these structures, where the entire scaffold was made from the hydrogel (i.e., the gels were not attached to a filter). A challenge in adapting these gels to the filter-bioreactor scaffold format is the substantial degree of swelling (>1.5X) these gels undergo following photopolymerization of the gel precursor solution. Swelling creates interfacial stresses that cause detachment of the gel from the filter or scaffold. We are addressing this by combining a previous observation that synthetic peptide gels functionalized with a dimeric RGD peptide, a heparin-binding domain from fibronectin, and EGF promote hepatocellular function (Mehta et al, 2010) with previous work using RGD-modified PEG gels of defined mechanical properties (Peyton et al, 2011) to create robust synthetic gels with defined adhesion functionalities. This is

an iterative process to identify conditions that provide appropriate adhesion functionalities, mechanical properties, and low degree of swelling. A second challenge is to create micropatterns of hydrogels on the filter substrates. One constraint is that regions of the filter must remain gel-free, to allow the flow of culture medium that provides both nutrient distribution as well as mechanical stress. Molding techniques that work well with impermeable substrates are not very amenable to use with porous filters, as the macromers flow into all regions of the filter. Photolithography, an alternative to molding, can be challenging when the feature sizes of the gel structures are relatively tall (>250um). We are modifying both of these approaches iteratively with new gel precursor formulations to control swelling and stiffness on filter-polymerized micropatterned gels.

Specfiically, in collaboration with Rashid Bashir (UIUC) a sterolithography approach based on photocrosslinking PEG precursors has been employed to create the scaffold features bonded to the semipermeable membrane. We first determined how to functionalize the membrane so that it would react with the PEG, but not leach residual chemicals following the processing. Initial gel fabrication protocols resulted in delamination of the gel from the membrane, a problem solved by pre-soaking the membrane in photo-initiator to prevent depletion of the photoinitiator from solution in the vicinity of the membrane. Gel scaffolds fabricated in this fashion have been used successfully at MIT to culture primary rat hepatocyte tissues in the bioreactor under normal flow conditions. Current efforts are directed at improving the reliability of seating the scaffolds in the flow path. This work is now transitioning to the University of Pittsburgh under the aegis of the NIH Microphysiological Systems Program. An additional feature of the bioreactor has been developed (oxygen sensing) to improve monitoring of the physiological status of the cultures; as cells are stimulated by inflammation, or tumor cells escape from dormancy and proliferate, oxygen uptake increases.

A significant constraint in bioreactor operation for analysis of escape from dormancy is controlling transport of oxygen, which is needed to feed the growing tumor. In homeostatic liver tissue in the bioreactor, a major constraint on reactor design is ensuring adequate renewal of oxygen depleted by respiring tissue. We tested 3 different designs to assess the effects of how oxygen transport is affected by the surface area of the air/liquid interface, and in turn, how the steady-state function of primary hepatocytes is affected by the steady-state oxygen concentration at the tissue inlet. Based on these experiments, we have determined a limit on minimum oxygen transfer and have a basis for building reactors for specific applications. Concomitant with these experiments, we developed a new method for measuring oxygen accurately and more economically with ruthenium probes. Measurement of oxygen is a highly informative means of assessing the proliferation of cells in the bioreactor.

In short the majority of the Objectives have been accomplished while others have been upgraded based on findings during the ongoing project.

KEY RESEARCH ACCOMPLISHMENTS:

- Defined cell isolation protocols
- Labeled tumor cells for tracking
- Coordinated bioreactor design with cell seeding
- Defined design parameters for bioreactor specifications based on oxygen delivery for tumor cells “escaping from dormancy” in the context of homeostatic liver.
- Defined 3 approaches to creating soft hydrogel scaffolds and demonstrated that soft hydrogel scaffolds bonded to semi-permeable membranes could be (a) incorporated into the bioreactor and (b) supported viable liver tissue formation (rat hepatocytes).
- Determined that liver parenchymal stress promotes carcinoma redifferentiation (MErT) and intercalation
- Found that activated endothelial cells promote carcinoma cell proliferation in a dedifferentiated

state (EMT)
• Modeled micrometastasis stability in a proliferative mode, and found it to be narrowly constrained between dying out and growing out.

REPORTABLE OUTCOMES:

Articles:

A Wells, YL Chao, J Grahovac, Q Wu, DA Lauffenburger (2011). Cell motility in carcinoma metastasis as modulated by switching between epithelial and mesenchymal phenotypes. Frontiers in Bioscience 16, 815-837. PMID: 21196205.

CM, Williams, G. Mehta, S.R. Peyton, A.S. Zeiger, K.J Van Vliet, **L.G Griffith** (2011) Autocrine-controlled formation and function of tissue-like aggregates by primary hepatocytes in micropatterned hydrogel arrays, Tissue Engineering, Part A 17:1055-68 PMID: 21121876

YL Chao, Q Wu, C Shepard, **A Wells** (2012). Hepatocyte-induced re-expression of E-cadherin in breast and prostate cancer cells increases chemoresistance. Clinical and Experimental Metastasis 29, 39-50. PMID: 21964676.

YL Chao, Q Wu, M Acquafondata, R Dhir, **A Wells** (2012). Partial mesenchymal to epithelial reverting transition in breast and prostate cancer metastases. Cancer Microenvironment 5, 19-28. PMID: 21892699.

NPAD Gunasinghe, **A Wells**, EW Thompson, HJ Hugo (2012). Mesenchymal-epithelial transition (MET) as a mechanism for metastatic colonisation in breast cancer. Cancer Metastasis Reviews, epub ahead of print. PMID: 22729277

D Taylor, JZ Wells, A Savol, C Chennubhotia, **A Wells** (2012). The dormancy dilemna: micrometastases are metastable during cellular proliferation. Submitted.

CONCLUSION:

The work on the proposal has been successful in both discovering a counterintuitive role of inflammation or stress of the parenchymal cells promoting carcinoma metastatic seeding and quiescent, while activation of the endothelial cells trigger carcinoma outgrowth. The work with the new soft-sided bioreactor armed with enhanced controls and oxygen measurements is set to generate a relevant model of micrometastatic dormancy and emergence ex vivo. The advances herein set the stage for an NIH-funded project entitled "All Human Microphysical Model for Metastasis Therapy" involving not only the three principles herein (Wells, Griffith and Stolz) but also groups from Draper Laboratories and a commercial entity, Zyoxel, committed to developing the bioreactor for widespread usage.

Cell Motility in Carcinoma Metastasis as Modulated by Switching between Epithelial and Mesenchymal Phenotypes

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Running title: EMT regulates Cell Migration

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Key words: Epithelial Mesenchymal Transition (EMT), Mesenchymal Epithelial reverting Transition (MErT), Growth Factor Receptors, Tumor Microenvironment, Migration, Tumor Dissemination

Abstract

The most ominous stage of cancer progression is the dissemination of carcinomas from their primary site into adjacent tissues, invasion, or distant ectopic organs, metastasis. These steps renders current extirpative therapies paliative at best and heralds the use of systemic therapies that are curative in only a small subset of patients even using the newest biological agents. This calls for a greater understanding of the tumor biology of tumor progression integrating the carcinoma intrinsic properties with the tissue environmental modulators of behavior. In no aspect of progression is this more evident that the critical step of tumor cell motility that is critical for both escape from the primary mass and seeding into ectopic organs and tissues. In this overview, we discuss how this behavior is modified by carcinoma cell phenotypic plasticity that is evidenced by reversible switching between epithelial and mesenchymal phenotypes. The intercellular linkages or absence thereof dictate the receptivity towards signals from the extracellular milieu. A number of clearly implicated such signals, soluble growth factors and cytokines and extracellular matrix components with embedded matrikines and matricryptines, will be discussed in depth. Finally, we will describe a new mode of discerning the balance between movement as an epithelioid cell and as a mesenchymal-like counterpart.

1. Introduction

Acquisition of a mesenchymal-like cell phenotype is one of the striking hallmarks of progression to dissemination of most all carcinomas. The one exception is ovarian carcinoma wherein spread throughout the peritoneal cavity occurs with an epithelial cell phenotype, but seeding of distant organs does coincide with cell dedifferentiation. This cancer-associated Epithelial-to-Mesenchymal Transition (EMT) has been strongly correlated with metastasis and shortened life expectancy of many carcinomas (1). As a number of interventions in animal models of tumor dissemination show a, at least partially, causal role for EMT in dissemination (2, 3), the question arises as to the cell behavior enabled. Herein, we will discuss the evidence that this EMT promotes tumor cell motility as the key event in progression.

Carcinogenesis involves a combination of mainly genetic events that generate a tumor cell. Though the source of that cancer is still uncertain for most carcinomas, for instance, whether the cancer derives from a stem cell compartment or by alteration of a differentiated epithelial cell, a series of mutations endows the cell with an ability to proliferate inappropriately to the situation. However, these intrinsic changes do not make the tumor cell fully autonomous. It is now appreciated that the tumor is a multicellular tissue in which non-cancer cells and the matrix modulate carcinoma behavior.

Further changes are needed for the cancer cell to disseminate from its origin. Despite many queries, the transition to this morbid and mortal stage appears not to be mutational. Rather, epigenetic events, possibly driven by the tumor microenvironment provide the cellular changes needed for dissemination. This suggests that unlike the mutational events that mark carcinogenesis, the alterations for dissemination are potentially reversible (4). Thus, we need to review the cellular aspects that mark escape from the ectopic site.

Carcinoma cell dissemination requires the acquisition of cellular properties and behaviors that enable the cells to escape from the original site, breach the surrounding barrier basement membranes and survive in ectopic locales. There are both qualitative and quantitative distinctions between localized invasion and distant metastasis. In the former, the cancer and support cells in the tumor may move together as a syncytium into the adnexia, providing not only for contiguous vascular support but also a quasi-orthotopic signaling environment. In metastasis, however, the cancer cells are generally accepted as solo travelers that must fully break from the primary mass and establish themselves in a truly foreign milieu; this is in addition to surviving the stresses of transiting vascular conduits. For cell intrinsic properties this dissemination requires quantitative degrees of changes.

The acquisition of the mesenchymal phenotype in carcinoma-associated EMT is a hallmark of carcinoma dissemination. Central to this is the downregulation of cell-cell adhesions mediated by E-cadherin (though N-cadherin is often found upregulated in many carcinomas allowing for cell heterotypic adhesion to endothelial cells for extravasation). This loss of cell adhesion may be partial to continue to provide syncytial behavior such as in localized invasion noted often in prostate carcinoma (5), or it may be complete to generate distant metastases such as in breast carcinomas (6).

The behaviors common to both forms of dissemination involve breach the basement membrane and active migration into an ectopic milieu. The initial steps involve recognition and remodeling of the matrix. While proteolytic activity is required for transmigrating this barrier (7, 8), it appears not to be a wholesale degradation but rather a selective processing (9, 10). Further, most carcinoma cells, whether invasive or not, present copious levels of proteases, so that the

regulation appears to be more one of activation or localized effect rather than de novo production (11, 12).

What is qualitatively different is autocrine and paracrine stimulation of cell motility. The loss of E-cadherin during EMT not only allows for cells to move away from the tumor mass but also is accompanied by a breakdown in the apical-basal polarity that separated apically secreted growth factors, often those for the EGFR and c-Met receptors, from their cognate receptors presented on the basolateral faces (Figure 1). This allows for autocrine stimulation of these motogenic receptors. This intrinsic cell behavior, by and large restricted to invasive and metastatic carcinoma cells (and cells during wound repair), is the focus of our discussion of the role of cell migration in carcinoma progression and how that reciprocally ties in with phenotypic switching.

2. Disruption of cell adhesion in EMT enables cell motility

One of the main distinguishing characteristics between epithelial and mesenchymal cells is that epithelial cells are linked by cell adhesion molecules to form contiguous sheets. These intercellular physical interactions not only limit motility away from the connected cells but also establish apico-basal polarity that regulates signaling between cells and with the surrounding environment. In contrast, mesenchymal cells exhibit transient and changeable front-back polarity and present loose and readily tractable intercellular contacts. This dictates that epithelioid cells act within a tissue whereas the cells in the mesenchymal state may disseminate.

There are four main types of cell-cell junctional molecules that connect epithelial cells. Tight junctions provide a barrier for solutes and small molecules along the apical surface of cells. Adherens junctions provide strong mechanical cohesion through connection to the actin cytoskeleton, but also control key signaling pathways through sequestration of catenins. Desmosomes also mediate intercellular contacts, but through anchorage to intermediate filaments. Gap junctions form intercellular junctions that allow the passage of ions and small molecules. In addition, integrins are cell-substratum adhesion molecules that are located on the basal surface of epithelial cells and facilitate interactions between the ECM and the cytoskeleton. Members of all these different families of cell adhesion molecules act in concert to contribute to a fully polarized epithelial phenotype.

EMT and aberrant regulation of adhesion molecules at the primary site

Loss of cell-cell adhesions is a critical step during EMT that allows for physical detachment of individual or groups of cancer cells from the primary tumor. This also allows for autocrine activation of signaling pathways that enable migration (Figure 2). EMT is most discernable at the invasive front of primary carcinomas and has been visualized as individual or a group of cells migrating into the surrounding tissue (13). Downregulation of cell adhesion molecules have been repeatedly documented to be associated with invasion and poor prognosis in many carcinomas. Therefore, cell adhesion molecules are an important mediator of the transformation between epithelial and mesenchymal phenotypes during EMT in metastasis. The disruption of cell adhesion and consequent induction of motility is a critical step in metastatic progression.

Tight Junctions maintain the apical-basal polarity of epithelial sheets. Epithelial cells are most commonly found as sheets of cells that line the surfaces of organs. An important function

of epithelia is to serve as a barrier to maintain tissue homeostasis. The apical distribution of tight junctions serves as a "gate" to limit the transport of ions, pathogens and small molecules and as a "fence" to restrict lipid and membrane proteins along the apico-basolateral axis (14). The net result is that most growth factor receptors are restricted to the basolateral surfaces while the epithelial-expressed growth factors are secreted through the apical side, with the tight junctions preventing autocrine activation.

A fully polarized epithelial phenotype requires the cooperation of tight junctions, adherens junctions, and desmosomes. Dissolution of tight junctions is an early event in EMT so it is not surprising that several tight junction components are disregulated in cancer progression. Expression of occludin in breast cancer cells decreases invasion and migration *in vitro* and *in vivo* (15). Similarly, levels of claudins are downregulated in invasive carcinomas and exogenous introduction of claudins increases adhesion and prevents migration and invasion (16, 17). The Par complex regulates tight junction signaling and its expression is also altered in invasive carcinomas. Not only is complex member Par6 required for TGFβ-dependent EMT but also disruption of this complex perturbs apico-basal polarity and stimulates chemotactic migration by stabilizing front-back polarity (18, 19).

Cadherins are a family of transmembrane glycoproteins that mediate calcium-dependent homophilic interactions. The classical members most widely studied are E-cadherin, expressed in epithelial cells; N-cadherin, R-cadherin, P-cadherin, and cadherin-11, expressed by mesenchymal cells; and VE-cadherin, expressed by endothelial cells. The structures of these cadherins differ mainly in the extracellular domain, which is responsible for the adhesive function. However, the cytoplasmic domain is highly conserved and binds to β-catenin and p120, which through binding to α-catenin link the cadherins to the actin cytoskeleton. Importantly, β-catenin is a nuclear transcriptional co-activator for the mitogenic LEF/TCF family of transcription factors, so sequestration of this molecule by cadherins prevents activation of downstream signaling pathways (20).

As the only cadherin expressed by epithelial cells, E-cadherin has been described as the "caretaker" of the epithelial phenotype and thus loss of E-cadherin is central to EMT (21). Downregulation of E-cadherin expression has also been correlated to the progression of most carcinoma (22, 23). Loss of E-cadherin is sufficient to increase the metastatic behavior of noninvasive breast cancer cells and is a rate-limiting step of the transition from adenoma to invasive carcinoma (24, 25). However, in this mouse model a complete EMT was not necessary, as vimentin and other mesenchymal markers were not expressed. Furthermore, use of a dominant-negative E-cadherin that resulted in the subcellular localization and prevented intercellular contacts was sufficient to induce the invasive phenotype, but expression of a constitutively active β-catenin was not (26).

E-cadherin is considered an invasion suppressor, as transfection of invasive E-cadherin-negative carcinoma cell lines with E-cadherin cDNA decreases invasiveness, which can be reversed after treating transfected cells with an anti-E-cadherin function blocking antibody (27). Perturbation of E-cadherin expression can promote cell motility in several ways. Physical adhesion promoted by E-cadherin prevents the dissociation and migration of cells. Alternatively, E-cadherin down-regulation results in release of β-catenin from the membrane, where it can then act as a transcription co-activator in signaling pathways such as Wnt. Studies using E-cadherin mutants suggest that the β-catenin binding function and not adhesion is responsible for the invasion suppression (28). In addition, loss of E-cadherin alone is not sufficient to drive β-catenin signaling, so it is likely that E-cadherin regulates the threshold of β-catenin signaling

(29).

Although down-regulation of E-cadherin has been shown to be sufficient to induce the changes in cell behavior downstream of EMT, in some cases expression of the mesenchymal cadherins can be sufficient or dominant. Downregulation of E-cadherin is often, but not always, accompanied by an upregulation of N-cadherin suggesting a cadherin switch in EMT (30). However, colocalization of both E-cadherin and N-cadherin has been observed (31). In addition, forced expression of N-cadherin in the absence of changes in E-cadherin has been shown to induce migration and invasiveness of cancer cells either through FGFR signaling or through interactions with N-cadherin expressed by the surrounding stromal cells. Similarly, expression of R-cadherin in BT-20 breast cancer cells leads to downregulation of E- and P-cadherins and induction of cell motility through sustained activation of Rho GTPases (32). Although seemingly contradictory, these studies suggest that E-cadherin and the mesenchymal cadherins may induce motility via different mechanisms intrinsic to the disparate functions of the cadherins.

Desmosomes define the third class of cell-cell adhesion junctions. Desmosomal components are also commonly downregulated in carcinomas and associated with the presentation of distant metastases, especially in cancers of the head and neck (32). Desmosomes are intercellular adhesion molecules that are anchored to the intermediate filaments in the cytoskeleton. They are composed of the desmosomal cadherins that have an extracellular domain that mediates cell-cell adhesion and a cytoplasmic domain that interacts with plaque proteins that bind to intermediate filaments. Loss of the plaque proteins plakophilin-1 and -3 have been shown to increase cell motility and metastasis of carcinoma cells (33, 34). Transfection of desmosomal components desmocollin, desmoglein, and plakoglobin into L929 fibroblasts resulted in intercellular adhesion and suppression invasion into collagen gels even in the absence of the assembly of full desmosome complexes with linkage to intermediate filaments (35). These studies suggest that desmosomes mainly act to prevent cell motility through physical cohesion.

Gap Junctions are cell adhesion complexes that mediate intercellular communication, rather than adhesion, through the exchange of ions and small molecules. These aqueous pores are composed of hexamers of connexins (Cx), which form a membrane-spanning pore. There are over 20 subtypes of connexins, with most variability in the subtypes occurring in the cytoplasmic domain (36). There is evidence that gap junctions may perform channel-independent functions, including effects on cell migration. Inhibition of cell motility of prostate carcinoma and melanoma cells is correlated with increased localization of Cx43 at cell-cell contacts (37). In contrast, transfection of Cx43 into HeLa and glioma cells increases invasion *in vitro* and metastasis of melanoma cells *in vivo* (38-40). There is still much controversy over whether connexin expression is pro- or anti-migratory and whether the function of gap junctions differs depending on the stage of the tumor.

Integrins are cellular adhesion molecules that couple the cell to the extracellular matrix. Not only do they provide anchorage to the actin cytoskeleton but also transmit signals, based on both clustering and integrin isoform. Integrins are composed of α and β subunits that form a heterodimeric complex to determine specificity to ligands. Some integrin heterodimers exhibit great promiscuity by binding to several different ECM components while others may recognize only unique ligands. Epithelial cells typically express the $\beta 1$ subunits, which recognize collagen and laminin, and the epithelial-specific $\alpha 6\beta 4$, $\alpha v\beta 3$ integrins (41). The integrins are critical for providing the substratum adhesion during motility. When integrins are knocked down, motility commensurate with the mesenchymal transformation is abrogated demonstrating the enabling

function of these "adhesion" molecules.

Several studies have documented the differential expression, distribution, and ligand affinity of integrins in preneoplastic lesions and carcinomas. Expression of integrins and therefore adhesion to ECM is regulated by TGF-β, which is a potent inducer of EMT (42). Induction of TGF-β in carcinoma cells activates the mesenchymal gene expression profile and promotes tumor invasion and spread (43, 44). TGF-β downstream targets Smads activate the expression of integrins and focal adhesion-associated proteins. Integrin signaling, through both αv, β1 and β5 integrins, has been shown to be necessary for TGF-β induction of EMT in mammary epithelial cells as addition of a β1 neutralizing antibody or β5 siRNA prevented invasion *in vitro* (45, 46). β5 integrin blockade did not influence mesenchymal gene expression such as the downregulation of E-cadherin, but prevented the formation of actin stress fiber with two-point focal attachment. The formation of these stress fibers may be necessary for the generation and maintenance of tension and force needed for cell migration (46). Transformation of mammary epithelial cells with the Fas oncogene induces EMT and the upregulation of integrins α2, α3, α5, α6, and β1 and consequently increased adhesion to matrix components collagen, fibronectin and laminin 1. EMT and integrin expression changes in these Ras transformed cells is maintained by an autocrine TGFβ1 loop (47). Besides changes in expression level, localization of integrins can also contribute to the capacity for cell migration in EMT. For example, expression of the α6β4 integrin in normal mammary epithelial cells is localized in hemidesmosomes to connect intermediate filaments with laminin in the basement membrane. However, in invasive breast carcinoma cells, α6β4 is localized to the lamellopodia of invading cells (48).

Loss of cell polarity leads to dysfunctional growth factor signaling

As described in the preceding sections, besides tethering cells together to prevent the detachment and migration of individual cells, tight and adherens junctions also serve to establish tissue polarity. This is a critical regulatory mechanism, as most all epithelial cells secrete growth factors from their apical surfaces, and also express the cognate receptors, but on their basolateral surfaces (Figure 1). When cell adhesion molecules are disrupted in carcinoma-associated EMT, this organization is lost, and the growth factor receptors that are located basolaterally can now come into unrestricted contact with their ligands. Furthermore, the growth factors now have access to the basement membrane, and stromal compartment, and can affect changes in the tumor microenvironment to further promote motility. For example, induction of EMT through TGFβ1 expression normally leads to increased ECM production and deposition and reformation of the basement membrane that stops the autocrine loop. However, in tumors this feedback loop is disrupted and TGFβ1 is produced continuously.

3. Motility in escape from primary site

Following the loss of cellular adhesion that allows for detachment from the primary tumor mass, tumor cells must penetrate through the surrounding tissue and basement membrane in order to disseminate. Intravital imaging has revealed that within primary tumors there are two categories of cells: very motile single cells and slower collectively moving cells; and that the mode of the cell motility determines spread through the blood or lymphatic system, respectively (49). Induction of EMT leads to a program of epigenetic changes to confer a migratory and invasive phenotype (50). In addition, tumor-surrounding stroma is actively remodeled by

proteolytic degradation of the extracellular matrix (ECM), which causes the release of growth factors and other molecules that provide feedback signals for further active cell migration (Figure 3). Dysregulation of matrix metalloproteinases contributes to tumor cell migration through multiple mechanisms: releasing individual cells by cleaving junction proteins, cleaving ECM to allow movement of the cells, unmasking cryptic sites with new roles in tumor cell migration, and releasing growth factors 'deposited' within the ECM, such as b-FGF, TGF-β1, PDGF, HB-EGF/Amphiregulin, and IFN-γ (51). Signaling towards motility is achieved both by receptors that modulate adhesion and provide basal traction and receptors for cytokines and growth factors (52).

Motility signaled from soluble factors

Cancer cells accumulate intrinsic/autonomous behaviors that allow for dysregulated growth, but despite these genetic mutations, they remain responsive to external signals in the form of growth factors. It appears that it is these signals that drive both EMT and the subsequent migration leading to dissemination that occurs of after neoplastic transformation generates the primary tumor growth. In growth factor-induced cancer invasion autocrine, paracrine, and matricrine loops (Figure 3) coordinately contribute to tumor progression. Paracrine loops function in both directions, with cancer cells driving stromal cells to alter the microenvironment and subsequent signals released by the stromal cells promoting cancer cell migration.

The first step in metastasis is cell motility in the primary tumor. Intravital imaging has revealed that within the primary tumors there are two categories of cells: very motile single cells that represent only a small portion and slower collectively moving cells; and that the mode of the cell motility determines spread through the blood or lymphatic system, respectively (49). Growth factors signaling in the primary tumors has the major role in cancer dissemination. In vitro, a large number of growth factors alone can induce both EMT and cell migration (EGF, VEGF, TGF-β, SDF) (reviewed in (53)). Below, we will highlight a few growth factors and other soluble factors that are unequivocably implicated in cancer cell invasion.

EGF and its receptor system is the most-extensively described growth factor system for induced cell motility. Autocrine EGFR activating loops are present in most all carcinomas, with the produced ligand being EGR or TGFα, depending on quantiative balance between receptor and ligand (54, 55). The motogenic pathways activated by EGFR activity have been delineated (reviewed in (56)). PLC-γ is immediately downstream in EGFR motogenic pathway (57) and cancer cell migration in response to EGF is promoted by PI3K and PLC dependent mechanisms (58). Phosphorylation and inactivation of FAK (59) and increase in urokinase and MMP9 are just some of the downstream effectors in this cascade.

HGF, mesenchymal derived cytokine and its receptor **c-**Met are overexpressed or amplified in many types of human cancer. This is a second receptor tyrosine kinase that not only drives cell scattering and induces EMT, but also actuates motility. Activation of c-Src, PI3K and PKC are crucial for HGF-induced cell motility and are accompanied by increased MMP activity (60). In breast cancer, activated c-Met receptor can even activate EGFR through c-Src activation (61). HGF signaling role in cancer motility is further underscored by presence of somatic c-Met mutations in metastatic carcinomas that confer motile-invasive phenotype of cancer cells (62). Of interest, HGF is a pro-growth factor that is activated only upon cleavage in the extracellular milieu. The uPA system that activates HGF is upregulated by EGFR signaling in prostate carcinoma, suggesting a further amplification of cell dissemination (63).

IGF-1; the Insulin-like growth factor 1-IGF receptor axis promotes cell motility by activating AKT and MAPK pathways (64). In addition to the above pathways, IGF-1 also promotes cell migration by phosphorylation of FAK and paxilin. It has been shown that IGF-1 stimulates cell migration coordinately with ECM integrin stimulation: IGF-1 can bind to vitronectin ,which is upregulated at the leading edge of migrating cells, and IGF-1-VN- IGF-Binding protein complexes promote cell migration by sustained activation of PI3K/AKT pathway (65). IGF-1 induced migration is mediated by increase in MMP-9 activity and αvβ5 integrin activation (66). IGF-1 induced PI3K/AKT signaling axis also promotes expression of MT1-MMP and synthesis of MMP2 and facilitates invasion of tumor cells (67).

TGF-β, is another growth factor that can alone induce epithelial to mesenchymal transition. TGF-β may have a role in the initial dissemination process. Fast moving single cells that are able to intravasate express high levels of TGFβ signaling (49); it is believed that transient high TGFβ activity in the primary tumor enables high metastatic efficiency at the primary site and that decreased TGFβ activity at the secondary site allows the resumption of the cell proliferation program (reviewed in (68)). In addition, paracrine TGF-β1 signaling induces ECM deposition (collagens, fibronectin, tenascin, elastin) by myofibroblasts thus promoting a pro-migratory microenvironment. This signaling system is distinct from the classical growth factor receptors noted above as it signals via serine/threonine kinases and SMAD intermediaries. There are three ligand isoforms with TGF-β1 being the one linked to cancer dissemination.

Cytokines/chemokines are soluble factors most often implicated in the inflammatory response, though they also signal to and from formed elements of tissues. Many cancers show evidence of active inflammation that appears to be supportive rather than anti-neoplastic (69-71). Cytokines, small proteins originally found secreted by specific cells of the immune system, which carry signals locally between cells to trigger inflammation and respond to infections, have also be revealed to be involved in tumor initiation and progression. In this section, the discussion will be mainly focused on migration signals from primary site in cancers via some cytokine receptors. One of the best described examples is that of the tumor associated macrophages (TAM) that appear to chemotax breast cancer cells in a reciprocal paracrine signaling with the cancer cells involving CSF-1 and EGF [Condeelis].

Tumor necrosis factor-alpha (TNF-α) was firstly identified as an anti-tumor cytokine by inducing immune-mediated necrosis of cancers (71). In recent years, evidences indicate TNF-α can also play an important role in prompting cancer cell migration and invasion (72). TNF-α is expressed in a variety of cancers, including lymphoma, breast, ovarian, pancreatic, renal, colon and prostate cancers (73-79). TNF-α induces breast and ovarian cancer cell invasion through activation of the NF-κB and JNK signaling pathways, following by elevation of MMP production in cancer cells (80, 81). Studies in ovarian cancer cells indicate that TNF-α also enhances cell migration and metastasis through induction of CXCR4 chemokine receptor via a NF-κB-dependent-manner (82). How CXCR4 regulates cancer cell migration will be discussed in the following context. Furthermore, TNF-α can also promote breast cancer cell trans-endothelial migration through upregulation of endothelial lectin-like oxidized-low-density lipoprotein receptor-1 (LOX-1) (83). Interestingly, both tumor- and macrophage-produced TNF-α plays an important role in the epithelial-mesenchymal transition (EMT) via repression of E-cadherin expression (84, 85). EMT in regulation of cancer cell motility and metastasis will be discussed under other subtitles.

SDF-1/CXCL12, the homeostatic chemokine *stromal cell-derived factor-1* is the only

chemokine for the widely expressed cell surface receptor CXCR4 (86). The CXCR4-CXCL12 axis regulates the migration of cancer cells to metastatic sites in many carcinomas (87-92). Blockade of CXCR4 signals using chemical antagonists, antibodies, or interfering RNAs inhibits tumor dissemination and metastasis in animal models (87, 93-96). The expression of CXCR4 can be regulated by VEGF and TNF in many cancers (82, 97). With binding of CXCL12 to CXCR4, the receptor activates phospholipase C (PLC) and phosphoinositide-3 kinase (PI3K) and inhibits adenylyl cyclase by different G-protein subunits (98). Signaling from PI3K induces the activation of PAK, Akt and RhoGTPase, which play important roles in cell polarization and actin polymerization involved in cell migration. On the other hand, PLC activates calcium release and protein kinase C (PKC), followed by Erk activation leading to cell migration (99-101). In addition, CXCR4-CXCL12 signals also direct invasion of human basal carcinoma cells and prostate cancer cells by the up-regulation of MMP-13 and MMP-9 respectively (102, 103). Cancer cell survival signals induced by CXCR4-CXCL12 axis will not be discussed here.

Motility signaled from the Matrix

The functional connection between properties of the extracellular matrix (ECM) and normal cell behavior in tissue homeostasis is well documented (reviewed in (104)). Though cancer cells are mutated and their responses dysregulated, they remain responsive to these same signals. Tumor progression is characterized by changes in ECM structure and composition, produced by both epithelial and mesenchymal cells and these changes influence the type of cell migration by providing ligands and the structural frame (reviewed in (105)). Apart from growth factors embedded within the ECM, modulation of migrational mode is achieved by varying the expression of adhesive and anti adhesive ECM proteins and proteolytic cleavage of the present ECM components.

Various ECM proteins with potential pro-migratory roles are upregulated in cancerous tissues: collagens I and IV, laminins, tenascin C, fibronectin and vitronectin. The ability to alter the stromal microenvironment correlates with the tumor invasive potential (106) (107) . Cancer cells induce gene expression changes in fibroblast and other stromal cells to produce ECM molecules that promote tumor migration and increase MMP production to loosen the stiffness of the matrix (108); (109). Depending on the cell surface receptors present, cancer cells may utilize different ECM components to improve the migration (110),(111). In addition, proteolytically cleaved fibronectin, laminin and collagen compete with their non-cleaved counterparts for adhesion sites and facilitate cell detachment (reviewed in (112)).

While the ECM signaling through integrins provides sensing of the mechanical properties of the ECM and basal adhesion and traction upon which growth factor pro-migratory signaling can function, another class of ECM molecules – matrikines and matricryptines – has emerged as crucial to cancer motility (113). Matrikines posses low binding affinity for growth factor receptors but are often present in high valency, which increases avidity for the receptor and enables signaling. These domains, to-date, are found in collagen, laminin, decorin and tenascin C and they enable persistent non-degradable signals. Presentation of growth factor-like sequences within constrained ECM results in unique signaling with preferentially activating pro-migratory cascades compared to soluble ligands (114).

Below, we will highlight a few of the ECM proteins that are dysregulated during epithelial to mesenchymal transition and contribute to cell motility signaling through both integrin signaling and tyrosine kinase receptors.

Collagen I is one of the main structural components of ECM, but fibrillar collagen not only serves as a substratum for integrins but also signals via DDR receptors. Up-regulation of collagen I in metastatic adenocarcinomas is mainly derived from the tumor stroma (115) and contributes to the increased stiffness of the tissue (116). Cancer cells tend to migrate toward the regions of the increased stromal stiffness (117), but loosening of the fibrilar collagen enables cancer invasion (118). MT1-MMP expression levels, the matrix-metalloproteinase mainly responsible for collagen I degradation, correlate with tumor invasiveness. The enhanced migration of cultured tumor cells in the presence of collagen degradation products and not intact collagen suggests a role for collagen fragmentation in tumor invasion (119) (120) suggest the role of collagen fragmentation in tumor invasion. This dual nature of collagen function is still to be understood, but it likely relates to quantitative balances of signals.

Collagen I can promote migration via $\alpha 1\beta 2$, $\alpha 5\beta 3$ or $\alpha V\beta 3$ integrin signaling and via discoidin domain receptors (DDR), a class of tyrosine kinase receptors (121) (122). DDRs are upregulated in many types of cancer (reviewed in (123) and *in vitro* overexpression of DDRs increases migration of cancer cells (124) (125). It has been shown that collagen I overexpression promotes motility by upregulation of N-cadherin expression through $\alpha 2\beta 1$ and DDR1 signaling (126) (127), which underlies the role of collagen I in EMT.

Laminin 5 (Ln-5) is one of the major components of the basement membrane. While epithelial cell adhesion to basement membrane occurs via integrin adhesion to laminin-5 (Ln-5), cleavage of Ln-5 by MT1-MMP (128) and MMP-2, both upregulated in tumors, reveals cryptic pro-migratory sites (129). These pro-migratory sites were shown to be EGF-like repeats that stimulate breast cancer cell migration in an EGFR-dependent manner (130). Up-regulation of Ln-5 was observed in many carcinomas, especially at the invasion fronts (131), which further supports role of the Ln-5 in dissemination from the primary site.

Tenascin C (TN-C) upregulation in invasive carcinomas recalls the similarities of cancer with embryogenesis and wound repair (onco-fetal-wound connection). In normal physiological TN-C establishes interactions between the epithelium and the mesenchyme during embryonic development, tissue differentiation and wound repair and its expression is transient and strictly regulated (132). Persistent high levels of TN-C are present in various tumor tissues, including brain, bone, prostate, intestine, lung, skin, and breast (reviewed in (133)) and are produced by both epithelial tumor and stromal cells (134). TN-C expression can be induced by various growth factors and cytokines (EGF, TGF-β, TNF-α, IFN-γ, IL) and by mechanical stress and hypoxia, all present in the tumor environment (reviewed in (135)) and its upregulation coincides with situations requiring either proliferation or migration.

TN-C is a multidomain molecule, and FNIII-like repeats of TN-C can interfere with integrin signaling, thereby enhancing cell proliferation (136), but soluble TN-C induces loss of focal adhesions and increase in cell migration, by binding to annexin II on cell surface through an alternatively spliced FNIII- like domain (137). In glioma cells, tenascin C promotes migration via $\alpha 2\beta 1$ integrins and has a positive effect on cell migration on fibronectin (138). Another way in which TN-C promotes invasion is by stimulating the production of matrix metalloproteinases: in chondrosarcoma, breast cancer and glioblastoma exogenous addition of the large splice variant of TN-C, TN-C320, or induction of its endogenous expression increases production of matrix metalloproteinases and invasion in *in vitro* assays (139-141).

Most recently, it has been shown that tenascin C possesses a novel mode of matricrine signaling via cryptic growth factor receptor ligands in its epidermal growth factor (EGF)-like repeats that are able to bind to the epidermal growth factor receptor (EGFR)(142, 143) as well as

to subsequently activate EGFR-signaled pro-migratory cascades in fibroblasts (144). Unlike in the case of soluble EGFR ligands where binding induces internalization and degradation upon binding to receptor (145), the EGF-like repeats of TN-C cannot be internalized and constantly signal from the cell membrane (144). EGF-like domain of TN-C can be released by MMP cleavage (146), and in the face of increased MMP activity during epithelial to mesenchymal transition TN-C mediated EGFR motogenic signaling is very possible.

Avoidance of Stop Signals

The migration of tumor cells from their primary mass to ectopic sites not only requires positive signals as noted above, but also avoidance of inhibitor signals. This takes two steps. The first of which is to down-regulate the molecules/structures that maintain organ structure. This is achieved during EMT, the hallmark of which is loss of E-cadherin homotypic interactions and connexin-43 gap junctions. However, this just sets the stage for motility. The positive signals have been described above, but the question remained of whether there are 'stop motility' signals that need to be overcome.

Recently, a 'stop motility' axis has been described for the physiological cell migration during wound healing (147-149). This operates via the CXCR3 receptor for the family of ELR-negative CXC chemokines. CXCR3 is activated by specific binding of the ligands, CXCL4/PF4, CXCL9/MIG, CXCL10/IP10, CXCL11/IP9/I-TAC, and the activation induces diverse cellular responses, including chemotactic migration and cell proliferation, or inhibition of migration and even endothelial death depending on the cell type (150). CXCL9, CXCL10, CXCL11 can be induced by INF while CXCL4 is released from alpha-granules of activated platelets during platelet aggregation (86). There are two splice variants of CXCR3, CXCR3A and CXCR3B; and CXCR3B contains longer extracellular domain at N-terminus (151). CXCR3A mainly functions in promoting cell proliferation and motility (151, 152). However, CXCR3B, primarily found expressed on fibroblasts, endothelial and epithelial cells, inhibits cell growth and migration (151, 153). Some studies have suggest that CXCR3A and CXCR3B play reciprocal roles through different G-protein coupling and lead distinct signaling transduction pathways (151, 154, 155) (156). Chemokines CXCL9, CXCL10 and CXCL11 bind to both CXCR3 isoforms, while CXCL4 only associates with CXCRB variant, possibly due to the extended extracellular domain of CXCR3B (151).

CXCR3 expression has been shown in human breast, colon, renal, and prostate cancer cells, as well as, human melanoma, breast, colorectal and renal carcinomas (157-165). Several study groups have reported CXCR3 promotes breast, colon and melanoma cell metastasis, but has no effects on tumor growth in murine models (161, 164-166), suggesting CXCR3 plays a more important role in tumor metastasis than localized expansion in these types of cancers. However, how CXCR3 regulates tumor growth and metastasis remains unclear. Since the CXCR3B isoform was identified recently, only a few studies have focused on the functions of two CXCR3 splice variants in cancers. Renal cells with calcineurin inhibitors treatment developed bigger tumors in nude mice by downregulation of CXCR3B, the expression of which correlates with tumor necrosis in renal cell carcinoma (160, 162), indicating CXCR3A and CXCR3B possibly has different influence on cancer progression *in vivo*. The studies of CXCR3 isoforms in keratinocytes, fibroblasts and endothelial cells suggest that in CXCR3 pathways, both CXCR3A and CXCR3B activate PLCβ by G proteins. PLCβ hydrolyzes the highly phosphorylated lipid phosphatidylinositol 4,5-bisphosphate (PIP2), generating two products: inositol 1,4,5-trisphosphate (IP3), a universal calcium-mobilizing second messenger; and

diacylglycerol (DAG), an activator of protein kinase C (PKC). IP3 induces intracellular calcium flux, which activates μ-calpain and results in cell motility induction. In addition to PLCβ activation, there is another unique signal transduction path via CXCR3B through an accumulation of cAMP. With CXCR3B signals, PKA, known as cAMP-dependent protein kinase, is activated which inhibits m-calpain activation and blocks cell migration (151, 153-155, 167). Therefore, in these cells, CXCR3A is likely to play a role of pro-migratory and CXCR3B signals as an anti-migratory signal for cell migration. However, how these two CXCR3 splicing variants regulate cell migration and invasion in cancers remains unclear. Our recent results suggest that prostate cancer cells increase CXCR3A and reduce CXCR3B expressions to subvert a stop signal to a promotion signal in cell motility and invasiveness regulations (168) (Figure 4).

4. Motility and Phenotype at the Target Organ

Extravasation from the vascular conduit and ectopic seeding are necessary for metastatic dissemination. Both of these steps require not only cell motility or at least transmigration but also cell-cell interactions that enable the cancer cell to interact with its new and foreign milieu. Carcinoma cells, unlike hematopoietic cells, are arrested due to size prior to extravasation, allowing more time for interactions and juxtacrine signaling to ensue that enables the carcinoma cell to squeeze between the endothelial lining. Further, once the vascular wall is breached, carcinoma cells have been noted to move towards the post-capillary spaces of the tissue, as if seeking a lower oxygen environment. This may reflect the glycolytic metabolism of carcinomas. The mesenchymal phenotype of disseminating carcinomas promotes all these steps. However, ectopic seeding may be a unique situation that will be discussed below.

Expression of adhesion molecules during extravasation

Once a cancer cell has undergone EMT to enable migration and dissemination from the primary tumor, a new set of challenges must be overcome in order to establish metastatic foci at a secondary organ site. Although mechanical entrapment of circulating tumor cells occurs, tumor cells must then actively adhere to the vascular and extravasate, or migrate through the endothelium into organ parenchyma. The process of extravasation is similar to diapedesis, or transendothelial migration, exhibited by leukocytes in inflammation. During diapedesis, leukocytes adhere to and roll along the vasculature and then migrate between endothelial cells. The initial attachment of cells to the endothelium is mediated by a class of cell adhesion molecules called selectins, followed by stronger adhesions facilitated by immunoglobulin adhesion molecules, integrins and cadherins. These are not merely attachments but signals between the endothelial and extravasating cells that direct retraction between the endothelial cells allowing for active movement of the invading cells through the vascular lining. Expression of many of these same cell adhesion molecules are necessary for extravasation of disseminated carcinoma cells (169).

Selectins are a family of adhesion receptors that bind to carbohydrate ligands. E-selectins are expressed primarily by endothelial cells, P-selectins by platelets, and L-selectins by leukocytes. Presentation of selectin ligands on cancer cells is believed to be critical to extravasation. Interactions of circulating cancer cells with platelets and leukocytes via P- and L-selectins may support tumor cell embolic arrest and immune evasion in the vasculature (170). Cancer cell binding to E-selectin on endothelial cells is critical to the extravasation of colon cancer cells in metastatic colonization of the liver. Attachment of cancer cells to the endothelium

and subsequent formation of metastases can be inhibited by addition of antibodies against E-selectin (171). Furthermore, selectin-dependent adhesion to endothelial cells results in morphology changes, reorganization of the cytoskeleton, and tyrosine phosphorylation, suggesting that these interactions are not limited to adhesion and may have downstream signaling effects (172). Differential expression of selectin ligands can also influence the site of metastastic colonization and account for organotropism (173).

Following the attachment initiated by selectin binding, adhesion between cancer cells and endothelial cells may be further strengthened by other adhesion molecules. Expression of immunoglobulin cell adhesion molecule (IgCAM) family members ICAM and VCAM has been observed in distant metastases of colorectal cancer but not in benign lesions, suggesting that these adhesion molecules are part of the EMT. The attachment of metastatic cells to endothelial cells and to extracellular matrix has been shown to be necessary for metastasis (174). The cadherin switch that occurs during EMT results in the down-regulation of E-cadherin and the upregulation of N-cadherin. Endothelial cells have been shown to express N-cadherin, so this switch may facilitate the heterotypic binding of cancer cells to endothelial cells. Indeed, N-cadherin has been shown to mediate attachment of MCF-7 breast cancer cells to endothelial monolayers as well as the transendothelial migration of melanoma cells (31, 175). Exogenous expression of Cx43 into MDA-MET, a breast cancer cell line variant that is highly metastatic to bone, results in increased adhesion to endothelial cells. Others have also shown similar heterophilic binding between cancer cells and endothelial cells melanoma and lung cancer (39, 176). Finally, engagement of integrins expressed on cancer cells also contributes to adhesion to the microvasculature, as antibodies against $\beta 1$, $\alpha 2$, and $\alpha 6$ integrins inhibited adhesion to and migration through sinusoids in colorectal metastases to the liver (177). Although cancer cells may arrest in capillaries due to size-restriction, these studies show that adhesion to endothelial cells is nonetheless a required step of extravasation.

Adhesion molecules during colonization

Despite the wealth of studies describing EMT in carcinoma cells *in vitro*, and the strong clinical association between loss of expression of adhesion molecules and invasion and poor prognosis, metastases often present a well-differentiated, epithelial phenotype, bringing into question whether EMT is reversible. It is well described that signals from the primary tumor microenvironment greatly contribute to induction of EMT at the primary tumor, so dissemination not only removes cancer cells from these signals but also exposes them to new ones at the secondary organ site. Furthermore, post-extravasation survival has been shown to be the rate-limiting step of metastasis (178) and most cancers seem to display a propensity to metastasize to a set of organs that can not be explained by circulation alone. Just as adhesion impacts the intravascular survival of a circulating cancer cell, intercellular adhesion between cancer cells and parenchymal cells can influence survival at the ectopic site (179).

While the mesenchymal phenotype that results from EMT may promote invasion and dissemination, there is evidence that metastatic colonization favors an epithelial phenotype. In bladder carcinoma, cell lines selected *in vivo* for increasing metastatic ability reacquire epithelial morphology and gene expression. When these cells are injected orthotopically, they show a decreased ability to colonize the lung when compared to the more mesenchymal parental cell line. However, when they are injected via intracardiac or intratibial inoculation, they show an increased ability to colonize the lung compared to the parental cell line (180). Therefore, while induction of EMT through loss of E-cadherin may promote tumor invasion and dissemination,

MET through E-cadherin re-expression may allow the metastatic cancer cell to complete the last steps of the metastatic process and survive in the new organ [Wells, CEM 2008; (6). When queried by pathology, a number of studies have shown that E-cadherin-expression metastases may derive from dedifferentiated, E-cadherin-negative primary carcinomas (181-185). Similarly, changes in β-catenin localization have been documented (186) and a study of breast cancer found increased expresson of Cx26 and Cx46 in metastatic lymph nodes compared to the primary tumors, with even positive foci originating from connexin-negative primaries (187).

The question remains whether the well-differentiated phenotype observed in metastases is the result of an expansion of epitheloid cells or from reversion of EMT – a transition back to an epithelial phenotype from a mesenchymal state (MErT). As E-cadherin down-regulation in invasive carcinomas is largely the result of promoter methylation and transcriptional repression, cancer cells can easily switch between epithelial and mesenchymal phenotypes. Promoter hypermethylation leading to E-cadherin suppression is dynamic and reversible and therefore re-expression in response to changes in the microenvironment is possible (188). As evidence of the phenotypic plasticity of cancer cells, PC3 prostate cancer cells cultured in 3D Matrigel form cell-cell contacts, tight junctions, and decrease in mesenchymal gene expression, suggesting that a change in tissue architecture is enough to induce such morphological changes (189). Work in our lab has shown that coculture of breast and prostate carcinoma cells with hepatocytes results in the re-expression of E-cadherin (6). *In vivo*, mice inoculated with E-cadherin-negative MDA-MB-231 cells also form E-cadherin-positive lung metastatic foci (190, 191). The basement membrane component laminin-1 may participate in re-expression of E-cadherin at the metastatic site (192). While these studies show that reversion through MErT is possible, they do not rule out the possibility of expansion of epithelial cells that have detached from the primary tumor.

Selective cellular adhesion may account for some of the organotropism exhibited by cancers. For example, breast cancer typically metastasizes to the lung, liver, bone, and brain, while colorectal cancer may metastasize to a different set of organs. Mechanical entrapment in the first capillary bed encountered does not explain the characteristic pattern of metastases (169, 193). E-cadherin re-expression could explain the propensity for breast cancer cells to metastasize to lung and liver, both lined with epithelia. Aberrant expression of osteoblast cadherin, also known as OB-cadherin and cadherin-11, on breast and prostate cancer cells, increases metastases to the bone by increasing migration and intercalation with osteoblasts (194, 195). Furthermore, there may be changes in integrin profiles of metastatic cancer cells to adapt to the new ECM compositions of the target organ. One group has shown that human melanoma cells express αvβ3 integrins to adhere to lymph node vitronectin, while breast carcinoma cells utilize α3β1 integrins to bind lymph node fibronectin (196). The αvβ3 integrin combination when expressed in breast and prostate cancer cells also contributes to bone-specific metastasis (197, 198). While selective growth and chemotactic honing are also critical mechanisms that contribute to site-specific metastasis, selective adhesion facilitated by these cell adhesion molecules is certainly important.

5. Migration in mesenchymal and epithelial phenotypes

It is generally considered that EMT is associated with a significant gain in cell motility, due to loss of stable E-cadherin-based cell-cell junctions (e.g., (199-201)). However, disparities in motility behavior between epithelial and mesenchymal forms of a carcinoma tissue may be

more nuanced and context-dependent than this dichotomous notion. Epithelia can exhibit efficient migratory behavior even while maintaining integrity of cell-cell interactions (202), and some invasive carcinomas penetrate adjacent connective tissue as multi-cellular aggregates (203). Thus, the issue at hand likely more concerns quantitative differences in key characteristics and molecular regulation of motility behavior for mesenchymal versus epithelial phenotypes rather than residing in a qualitative "on/off" motility switch. One interesting model, in fact, suggests that cooperation between epithelial and mesenchymal subpopulations of a tumor enhances distal metastasis because contributions of motility from both are needed in order to meet diverse challenges inherent in intravasation and extravasation (3).

The mechanistic basis for mesenchymal and epithelial motility must emphasize different balances among the underlying biophysical processes of membrane (lamellipod, filopod, and/or invadopod) protrusion, cell/matrix attachment formation, cytoskeletal contraction, cell deformation, and cell/matrix detachment, along with cell/cell adhesive interactions. Cell/cell adhesive interactions are more important for coordinated epithelial cell aggregate motility, whereas lamellipod protrusion should have greater influence on individual mesenchymal cell motility; in both cases, nonetheless, net cell locomotion can only arise from an appropriate balance of forces associated with the cohort of biophysical processes involved. Indeed, computational models have been proposed for purposes of quantifying the relevant balance of forces generating net locomotion in each of the categories (204, 205), although a direct comparison has not yet been undertaken.

With respect to particularly vital processes, invadopodia associated with focal proteolysis of the extracellular matrix are believed to be generally vital for tissue penetration and highly prevalent in mesenchymal cells (206, 207). Nonetheless, at least some mesenchymal cell types appear to undertake locomotion in an amoeboid manner independent of matrix proteolysis under certain circumstances (208). Detailed quantitative biophysical and biochemical analyses of cell and matrix properties are beginning to elucidate the conditions under which proteolysis is critical or not (10, 207, 209). As with motility per se, the role of proteolysis in mesenchymal versus epithelial migration may not be categorical, with it contributing to both kinds of invasion (210). A very recent study has identified a set of pseudopod-specific proteins associated with metastatic tumor cell lines, with a subset (AHNAK, septin-9, eIF4E, S100A11) found to be essential for actin polymerization and pseudopod protrusion related to *in vitro* invasiveness (211). More established promoters of carcinoma invasion and metastasis involved in control of lamellipod and invadopod formation, at least in breast tumors, include a splicing isoform of the Ena-VASP protein Mena (212, 213), cofilin (214), and cortactin (215), among others. Motility and invasion can be governed not only by processes occurring at the cell front, of course, but also by other processes transpiring at the cell rear; calcium-independent calpain-mediated deadhesion of cell/matrix attachments has been found to be a process rate-limiting for migration and invasion of prostate tumor cells (216). It should be noted that calpain activity may also be involved in another biophysics process involved in invasive motility, by regulation of invadopodia (217).

Coordination of the underlying biophysical processes to produce cell migration depends intricately on integration of receptor-mediated signals distributed across multiple pathways, in both temporal and spatial manner (218), so it can be expected that mesenchymal and epithelial motility modes should exhibit diverse dependencies on various intracellular signals. Unfortunately, there are few literature reports bearing on this issue. We note that the question of signals driving motility of mesenchymal cells versus epithelial cells is not the same as the question of signals inducing epithelial-to-mesenchymal transition; the latter question has been

intensely investigated (e.g., (200, 219)) whereas the former question has seen little address to date. An intriguing clinical observation highlighting the point is the resistance of mesenchymal carcinomas to tyrosine kinase inhibitors of EGFR in contrast to the sensitivity of epithelial carcinomas to this set of drugs (53, 220-222). Since EGFR remains substantively expressed on the mesenchymal tumor cells, a major challenge is to determine how the signaling network governing motility becomes "rewired" during EMT. An analogous challenge for HER2-overexpressing breast epithelial cells has been addressed using quantitative phospho-proteomic measurement across multiple signaling pathways coupled with computational modeling to ascertain the key network differences between normal and overexpressing cells (223). A number of differences were elucidated, and computational modeling showed that quantitative combinations of a subset of pathway activities could predict the change in motility behavior as well as in response to various kinase inhibitors (224, 225). Some of us (AW, DL) have recently applied a similar approach to explore differences in motility-related signaling in mesenchymal versus epithelial forms of human mammary epithelial cells with EMT induced by the transcription factor Twist. This study has found that at least a half-dozen intracellular kinase pathway activities are differentially influential for mesenchymal versus epithelial motility across stimulation by a variety of growth factors including EGF, HRG, HGF, IGF, and PDGF, demonstrating the complexity of signaling network "rewiring" downstream of EMT induction (HD Kim, A Wells, FB Gertler, DA Lauffenburger; unpublished data).

6. Future directions

It is too early in our investigations of how the microenvironment interacts with carcinoma intrinsic changes to dictate tumor behavior and progression to propose interventions. Due to limitations of focus and space we have discussed only cell migration, which has been shown to be a critical step in the stage of tumor dissemination. We have not delved into proliferation or death (apoptotic, necrotic, or autophagic) as these cancer-associated dysregulation of these behaviors arise well before dissemination at the earliest stages of carcinogenesis. Also, the foregoing, while quite extensive, did not deal with critical aspects of immune response, systemic hormonal/cytokine signaling, and angiogenesis. Each of these whole organism responses impact upon tumor outcome in nuanced and situation-dependent manners, and hold avenues for successful interventions. Still, we posit that better examination of the local tumor microenvironment, at both the primary and ectopic sites, can highlight key regulatory, and possible targetable, events in the transitions to dissemination.

It is evident that more systematic approaches to these questions are needed. The variety of signals and possibilities of behavioral outcomes make it evident that no one signal is required and thus models must account for redundancy. Further, quantitative aspects will dictate the resultant behavior; a glaring example of this is collagen wherein this provides substratum traction for migration with higher concentrations providing a stiffer matrix that promotes mesenchymal phenotypic behaviors, yet collagen can serve as a barrier to dissemination. Further, the systematic approaches need to account for higher levels of regulation. Tissue- and site-specific signals determine which cell behaviors promote tumor progression. For instance, EMT resulting in mesenchymal-like single cell properties enables escape from the primary tumor mass but a reversion to epithelioid syncytial properties may be critical for ectopic survival once a distant site is involved. Further, invasion into adjacent tissues may be accomplished as a mass of mixed carcinoma and orthotopic stroma whereas distant metastases most likely involves isolated

cells adapting to the foreign environment.

Even within such consideration of multiple levels of control, and quantitative and nuanced analysis of the data, there remain areas for reductionist examination. One aspect that is only now becoming approachable is how the tumor cells escape from the physiological controls that maintain differentiation prevent epithelial mislocalization. While avoidance of the immune response was been appreciated for half a century, the signaling focus for the past decades has mainly highlighted acquisition of signaling capabilities the promote carcinoma progression. Yet, these carcinoma cells must overcome the physiological 'stop' signals that efficiently prevent the transient EMT of wound repair or embryogenesis from leading to dysplasia, and even in the most aggressive appearing carcinomas make dissemination a rare event at the cellular level. Yet, this is not likely to simply be avoidance by negation, but rather a switch in receptivity, as noted by the well-described dual nature of TGF- β signaling, a paradigm being seen again in early studies on the chemokine receptor CXCR3.

In sum, the understanding of carcinoma cell events that lead to the migration that enables dissemination is ripe for explorations at system and reductionist levels. These studies will likely yield not only fundamental insights into the multicellular and multicompartment tissue we refer to as a tumor, but also suggest avenues for interventions that target distinct stages in carcinoma progression to dissemination.

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Figure Legends

Figure 1. Transition from Epithelial to Mesenchymal Phenotype. EMT results in the loss of cell-cell adhesions allowing for the autocrine stimulation as the basolaterally-restricted receptors are no longer isolated from the apically-secreted motogenic cognate growth factors.

Figure 2. Key Motogenic Intracellular Signaling Pathways Emanating from Cell Surface Receptors. Shown are select receptors and the key motogenic signaling pathways. Not shown, for clarity of the schematic, are all the overlapping signals and other, less thoroughly documented pathways that have been linked to driving motility. These pathways have been demonstrated to be viable targets limiting tumor cell motility in preclinical models.

Figure 3. Matrix-Embedded and Encoded Signals Liberated by Proteolysis. Extracellular matrix is not only recognized by adhesion and other (DDR, etc) receptors but also contains predeposited soluble factors, encoded factors, and cryptic signaling elements. Proteolysis of the matrix, mainly by matrix metalloproteinase (MMP), degrade invasion inhibitors (I) and release and uncover these signals (GF – growth factors, MK – matrikines). Invadopodial-localization of such activity can be accomplished by membrane-tethered MMP. Adapted from (56).

Figure 4. CXCR3 Isoforms Modulate Motility in Opposing Directions. CXCR3A signaling mainly via Gαq subunits activates phospholipase C-β (PLC-b) to initiate calcium influx; activation of calpain 1 at the membrane shifts the adhesion regiment to a more permissive state to facilitate motility. CXCR3B, while also signaling via Gαq subunits, more strongly initiates Gαs subunits that trigger protein kinase A (PKA) to inhibit calpain 2 and prevent rear release during motility. In normal prostate epithelial cells, only CXCR3B is expressed, but in prostate carcinoma cells, both isoforms are present at roughly equivalent levels.

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Figure 1

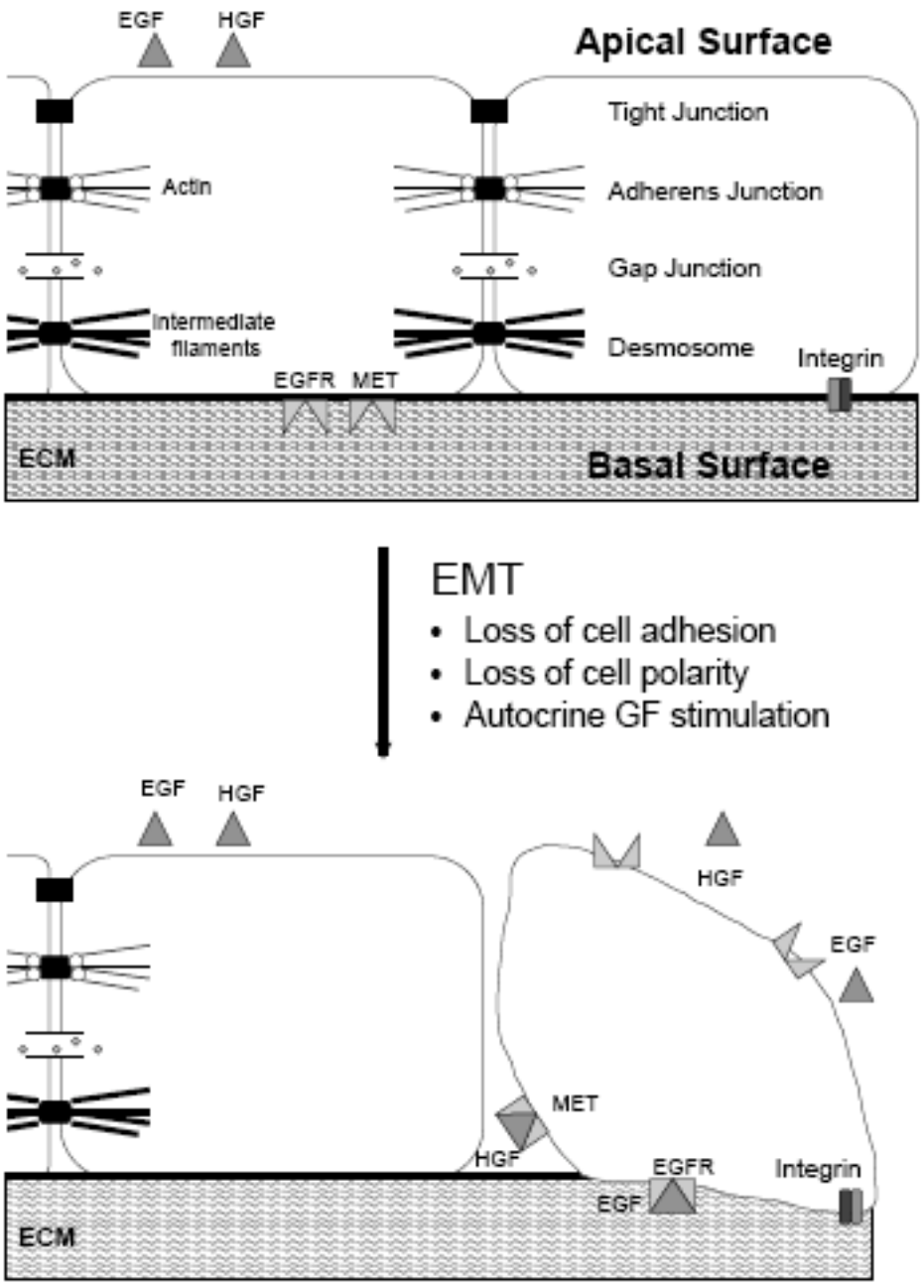

Figure 1. Transition from Epithelial to Mesenchymal Phenotype. EMT results in the loss of cell-cell adhesions allowing for the autocrine stimulation as the basolaterally-restricted receptors are no longer isolated from the apically-secreted motogenic cognate growth factors.

Figure 2

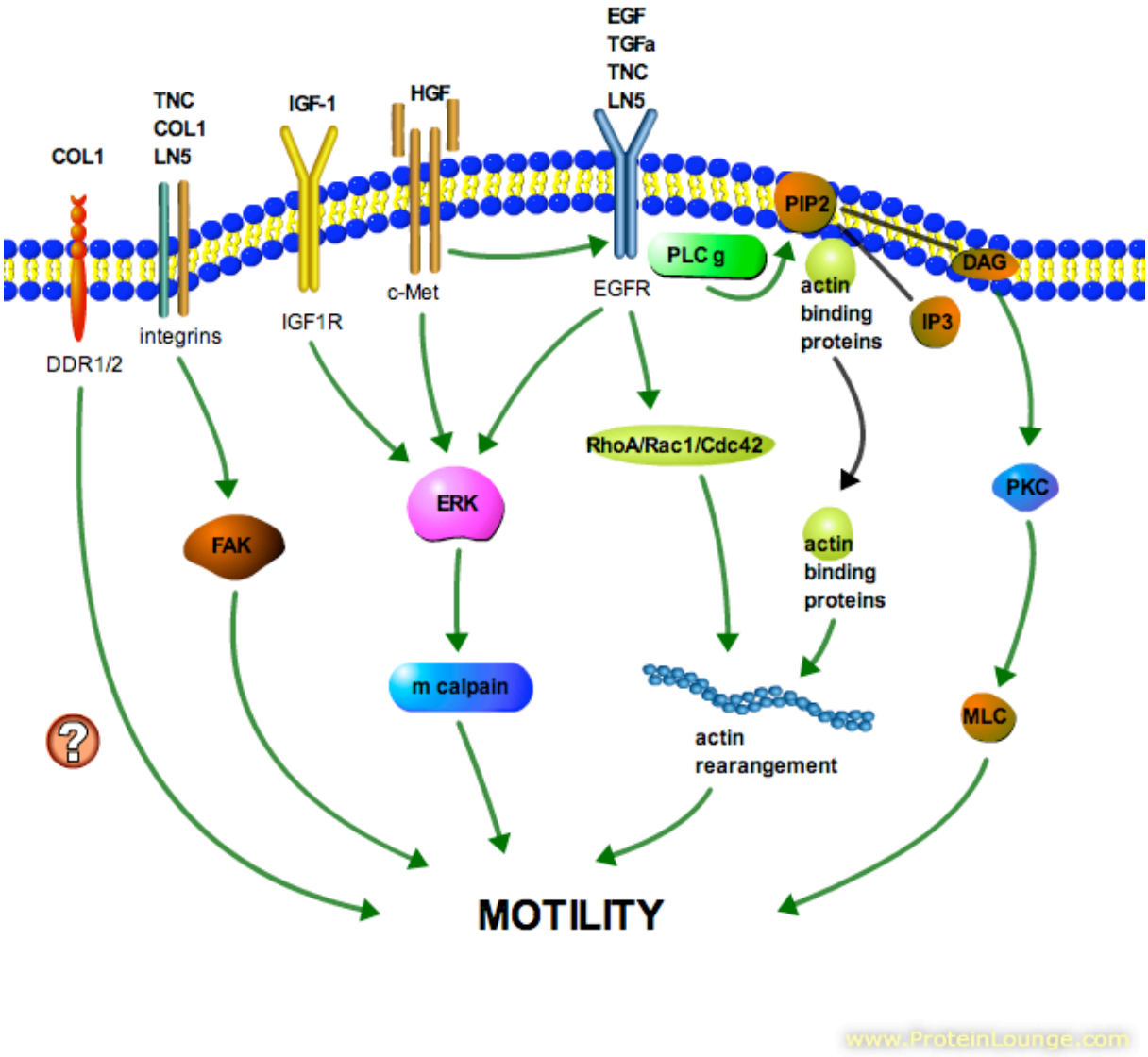

Figure 2. Key Motogenic Intracellular Signaling Pathways Emanating from Cell Surface Receptors. Shown are select receptors and the key motogenic signaling pathways. Not shown, for clarity of the schematic, are all the overlapping signals and other, less thoroughly documented pathways that have been linked to driving motility. These pathways have been demonstrated to be viable targets limiting tumor cell motility in preclinical models.

Figure 3

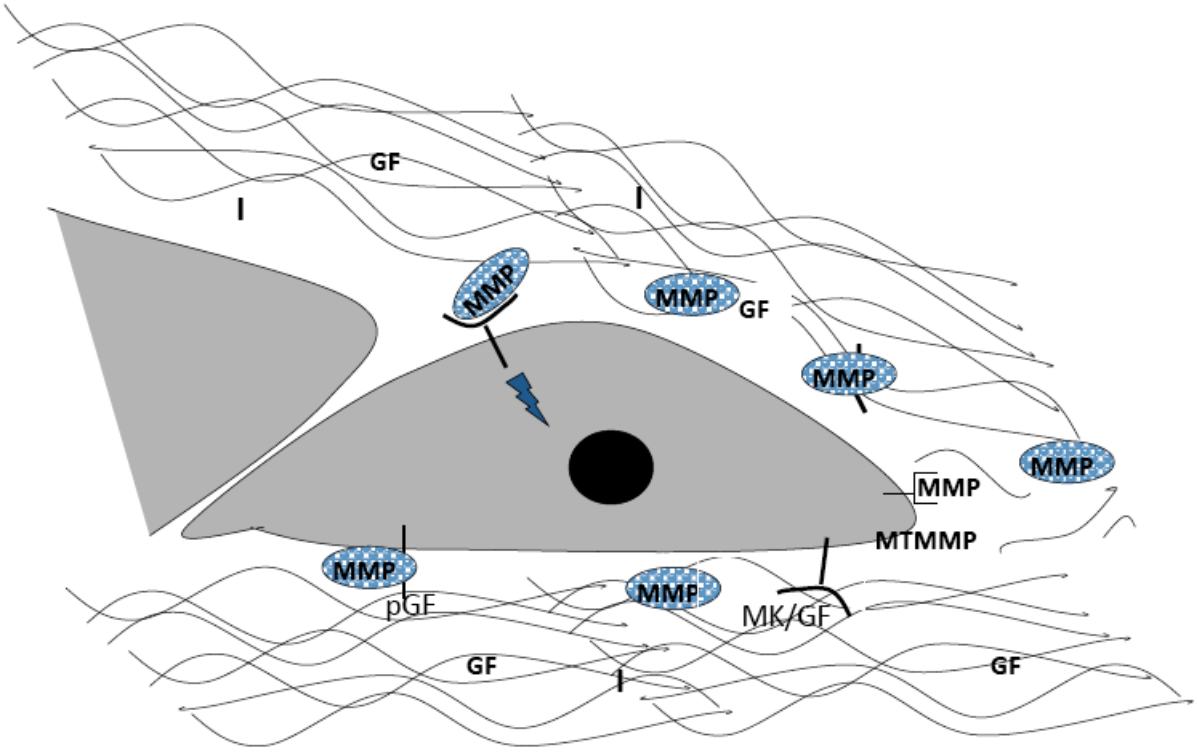

Figure 3. Matrix-Embedded and Encoded Signals Liberated by Proteolysis. Extracellular matrix is not only recognized by adhesion and other (DDR, etc) receptors but also contains predeposited soluble factors, encoded factors, and cryptic signaling elements. Proteolysis of the matrix, mainly by matrix metalloproteinase (MMP), degrade invasion inhibitors (I) and release and uncover these signals (GF – growth factors, MK – matrikines). Invadopodial-localization of such activity can be accomplished by membrane-tethered MMP.

Figure 4

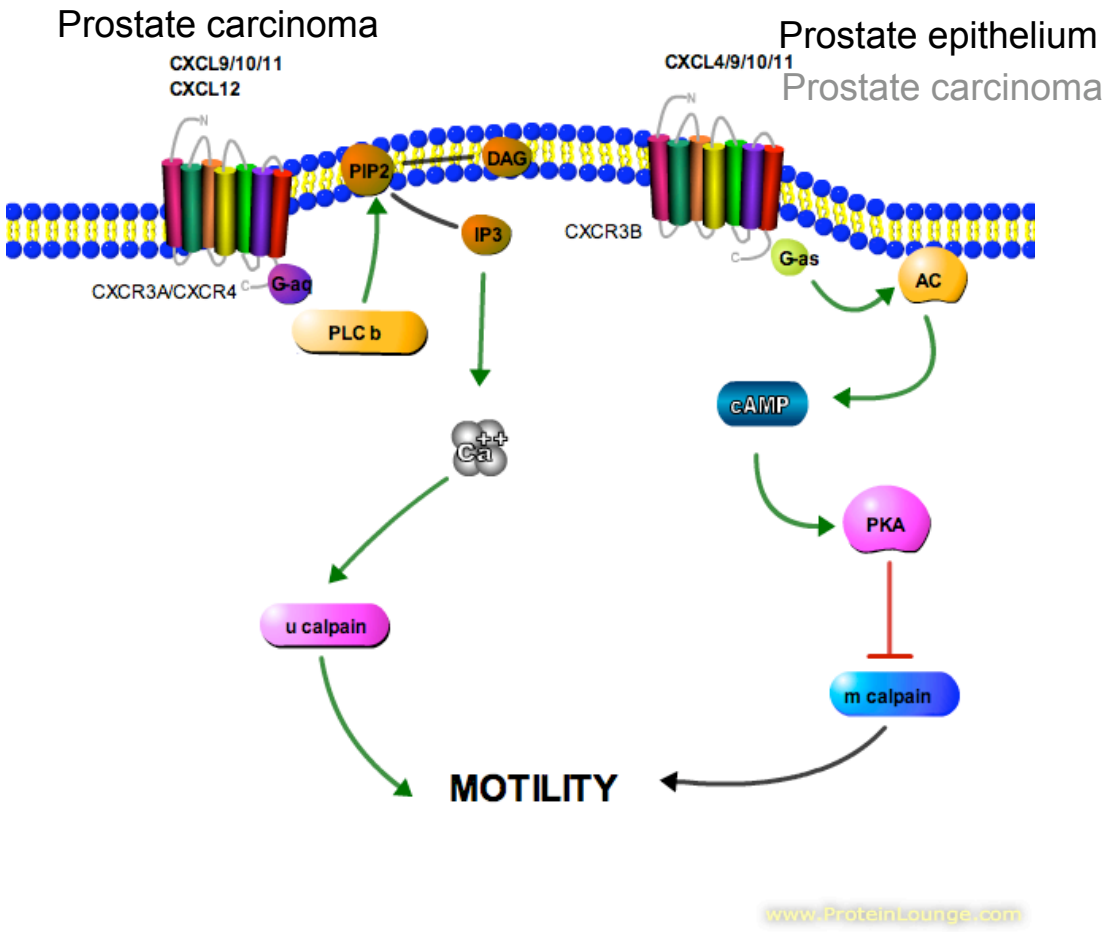

Figure 4. CXCR3 Isoforms Modulate Motility in Opposing Directions. CXCR3A signaling mainly via Gaq subunits activates phospholipase C-b (PLC-b) to initiate calcium influx; activation of calpain 1 at the membrane shifts the adhesion regiment to a more permissive state to facilitate motility. CXCR3B, while also signaling via Gaq subunits, more strongly initiates Gas subunits that trigger protein kinase A (PKA) to inhibit calpain 2 and prevent rear release during motility. In normal prostate epithelial cells, only CXCR3B is expressed, but in prostate carcinoma cells, both isoforms are present at roughly equivalent levels.

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Autocrine-Controlled Formation and Function of Tissue-Like Aggregates by Primary Hepatocytes in Micropatterned Hydrogel Arrays

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The liver carries out a variety of essential functions regulated in part by autocrine signaling, including hepatocyte-produced growth factors and extracellular matrix (ECM). The local concentrations of autocrine factors are governed by a balance between receptor-mediated binding at the cell surface and diffusion into the local matrix and are thus expected to be influenced by the dimensionality of the cell culture environment. To investigate the role of growth factor and ECM-modulated autocrine signaling in maintaining appropriate primary hepatocyte survival, metabolic functions, and polarity, we created three-dimensional cultures of defined geometry using micropatterned semi-synthetic polyethylene glycol–fibrinogen hydrogels to provide a mechanically compliant, nonadhesive material platform that could be modified by cell-secreted factors. We found that in the absence of exogenous peptide growth factors or ECM, hepatocytes retain the epidermal growth factor (EGF) receptor ligands (EGF and transforming growth factor-α) and the proto-oncogenic mesenchymal epithelial transition factor (c-MET) ligand hepatocyte growth factor (HGF), along with fibronectin. Further, hepatocytes cultured in this three-dimensional microenvironment maintained high levels of liver-specific functions over the 10-day culture period. Function-blocking inhibitors of α5β1 or EGF receptor dramatically reduced cell viability and function, suggesting that signaling by both these receptors is needed for *in vitro* survival and function of hepatocytes in the absence of other exogenous signals.

Introduction

Autocrine growth factor signaling by hepatocytes in the liver is a central mechanism for both physiological homeostasis and pathophysiological response of hepatocytes to stresses including inflammation and surgical resection. Epidermal growth factor receptor (EGFR) ligands, including transforming growth factor-α (TGF-α) and EGF, are produced by hepatocytes as well as nonparenchymal cells, and concentration gradients of these molecules influence a variety of cell behaviors in the intact liver.[1] Extracellular matrix (ECM) molecules can also serve autocrine functions; for example, hepatocytes produce plasma fibronectin (FN) while also expressing its receptor α5β1 integrin and the coreceptor syndecan-4.[2] FN fills the Space of Disse within the liver sinusoid, offering an adhesive matrix while allowing hepatocytes access to the circulating blood.[3] Thus, *in vivo*, hepatocytes manipulate their microenvironment via production of autocrine factors essential to their survival and function, in dynamic response to external cues.

Exogenous EGF or TGF-α is typically added to serum-free culture media to enhance hepatocyte survival and function, especially in 2D culture formats,[4–9] and EGF enhances spontaneous formation of three-dimensional (3D) spheroidal aggregates of adult hepatocytes cultured on minimally adhesive 2D substrates.[10–13] Hepatocytes in 3D spheroidal floating aggregates secrete ECM and exhibit long-term maintenance of liver functions[11,12,14–19] compared with cells cultured in 2D formats, wherein addition of DMSO or other cell types is required for functional maintenance.[20]

Unlike insulin and other hormones added to serum-free cell culture media, hepatocytes produce autocrine EGFR ligands *in vitro*.[6,21] The need for additional exogenous EGFR ligands in 2D culture, or in the formation of spheroids from cells seeded on 2D culture substrates, may arise from a failure of autocrine ligands to be retained locally; that is, ligands produced by cells in 2D can readily diffuse into the medium before recapture by cell surface receptors.[22]

The contribution of autocrine ligands to enhanced maintenance of liver-specific functions in 3D spheroidal cultures of hepatocytes is unclear. Spheroidal cultures are often characterized by a layer of cells with flattened morphology at the outer rim,[23,24] a phenomenon that may arise from gradients in diffusible autocrine growth factors (which may

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accumulate in the interior portion of the spheroid but diffuse freely from the surface into culture medium); from mechanical stresses associated with contraction and compaction of cells against a free surface; or from loss of appropriate ECM, such as FN, at the outer boundaries.

Here, we investigate the roles of autocrine growth factors and ECM in fostering viability, function, and polarization in adult rat hepatocytes using an experimental micromolded gel system that controls the geometry of 3D cell aggregation, presents a diffusion barrier to loss of autocrine factors, provides an initially inert (to cell adhesion) template that can be altered by ECM deposition to engage cells mechanically with the substrate, and provides a mechanically compliant environment that approximates the compliance of liver. Although collagen gels are serviceable for hepatocyte culture[20,25] and have been widely used to create micropatterned environments for other cell types,[26–28] hepatocyte adhesion to collagen via collagen-binding integrins induces cell signaling and phenotypes that might mask the effects of autocrine factors or drive cells to undesirable phenotypes.[25,29,30] To foster engagement of cell adhesion by cell-secreted FN, while dampening adhesive interactions during initial stages following cell seeding, we therefore focused on a semisynthetic gel comprising polyethylene glycol (PEG) linked to denatured fibrinogen (PEG–fibrinogen). Unlike endothelial cells and other αvβ3 integrin-expressing cells, hepatocytes do not bind directly to fibrinogen but may interact with fibrinogen indirectly through FN, which both binds to fibrin and is also recognized by α5β1 receptors on hepatocytes.[17,31] As fibrinogen is denatured during the process to form PEG–fibrinogen gels, the tertiary structure, which typically prevents fibrinogen–FN interaction, is lost and the secondary structure of fibrinogen peptides characteristic of the native protein is evident in the PEG-modified state,[32] and hence, presumably, it can bind FN. PEG–fibrinogen gels are relatively easy to micromold via UV-crosslinking processes and can be fabricated with mechanical properties comparable to liver[33–35]; hence, these gels provide an attractive experimental system for controlling hepatocyte aggregation and local retention of ECM produced by hepatocytes. Their permeability and degradation properties can also be tailored over a wide range through inclusion of additional PEG diacrylate with the PEG–fibrinogen macromer in the polymerization process.[36]

Exploiting these advantages of micropatterned PEG–fibrinogen hydrogels, this study probes the nature and action of autocrine loops in regulating survival and function of primary hepatocytes. Using a combination of immunostaining and function-blocking inhibitors, we demonstrate that primary hepatocytes in micromolded PEG–fibrinogen gels retain autocrine growth factors (EGF, hepatocyte growth factor [HGF], TGF-α) and cell-derived matrix (FN). These factors are necessary and sufficient to maintain hepatocyte differentiation in culture, as determined by cell morphology and metabolic function. This culture system may prove useful for analysis of other aspects of hepatocellular biology or as a platform to study hepatocyte metabolism of pharmacological agents.

Materials and Methods

Culture of primary hepatocytes in micromolded gels

Primary cells were isolated from male Fisher rats weighing between 150 and 250 g as previously described.[20] Following isolation, primary hepatocytes were resuspended in serum-free hepatocyte growth medium (HGM; low-glucose DMEM supplemented with 4 mg/L insulin, 100 nM dexamethasone, 0.03 g/L proline, 0.1 g/L L-ornithine, 0.305 g/L niacinamide, 2 g/L D-(+)-galactose, 2 g/L D-(+)-glucose, 1 mM L-glutamine, 50 mg/L gentamicin, 54.4 μg/L $ZnCl_2$, 75 μg/L $ZnSO_4 \cdot 7H_2O$, 20 μg/L $CuSO_4 \cdot 5H_2O$, 25 μg/L $MnSO_4$) without EGF.[37] Cells were plated onto micromolded gels at a density of 100,000 cells/cm^2 (720,000 cells/mL) in a 12-well tissue culture dish and spun at 100 *g* for 3 min; this process was repeated twice. After seeding, gels were transferred to new wells with fresh HGM to remove excess cells. HGM was replaced after 24 h and again after every fourth day of culture. Cells were incubated at 37°C under 5% CO_2 and 95% humidity for the time periods indicated; EGF was included in cultures at 10 ng/mL as indicated. For experiments with cyclic arginine–glycine–aspartate (cRGD) peptide, 10 μM of the peptide cyclo(Arg-Gly-Asp-D-Phe-Val) c(RGDfV) was included at the time of plating and maintained during culture (Peptides International). For experiments with EGFR inhibitor, 10 μM of the monoclonal antibody mAb225 against EGFR was included at the time of plating and refreshed at each medium change (kind gift from H. Steve Wiley Lab, PNNL). Twenty-four-well plates adsorbed with collagen I (BioCoat; BD Biosciences) with EGF containing HGM were used as controls in some experiments.

Synthesis of PEG–fibrinogen

PEG was acrylated similar to previously published protocols.[38,39] Briefly, acryloyl chloride (Alfa Aesar) was reacted with PEG-diol (6 or 10 kDa; Sigma) at 4× molar excess to available alcohol groups in benzene and under nitrogen pressure in the presence of triethylamine (Sigma) overnight at room temperature with protection from light. The resulting PEG-diacrylate (PEGDA) product was purified by multiple rounds of diethyl ether precipitation, vacuum dried, lyophilized, and stored under nitrogen gas and at −20°C. Acrylation efficiency was determined by H-NMR and ranged from 85% to 95%.

Fibrinogen was PEGylated by reacting PEGDA (10 kDa) with full-length fibrinogen in 4× molar excess to the number of cysteines on fibrinogen at room temperature in 8 M urea in phosphate-buffered saline (PBS), pH 7.4, for 3 h in the presence of tris(2-carboxyethyl)phosphine hydrochloride (Sigma).[39,40] The PEGylated fibrinogen product was precipitated in acetone and dialyzed for 24 h over three changes of PBS at 4°C. The fibrinogen content of each batch of material was quantified with a BCA protein assay (Pierce Chemicals), and the PEG content was quantified by weighing the lyophilized product and subtracting out the contributions of PBS and fibrinogen.[39] The fibrinogen content was 7.2 ± 0.2 mg/mL and the PEG content was 29 ± 3.3 mg/mL (both mean ± standard error).

Micromolding of PEG–fibrinogen gels

Microwell patterns, a 5×7 array of 500-μm-diameter circles, were designed using AutoCAD (Autodesk). A transparency mask was created from the CAD design and printed using a high-resolution printer (PageWorks). The transparency mask was used in photolithography of SU-8 photoresist to create 200 μm high patterns on the silicon wafer master.

Micropatterned stamps were made by replica molding of polydimethylsiloxane (PDMS; Sylgard 184) and curing the degassed elastomer mix (10:1, base:curing agent) over the silicon master overnight in a 60°C oven. Polymerized PDMS micropatterned stamps were peeled off the silicon master and used for patterning the PEG–fibrinogen hydrogel; 100 μL of a premixed solution containing PEG–fibrinogen (3.6%, wt/vol), 5% PEG-DA ($M_W = 6$ kDa) and 0.2% Irgacure 2959, was placed on the PDMS stamp, covered with a polycarbonate support scaffold,[41] and cured under a handheld long-wave UV lamp, B-100YP, (UVP) for 4 min. Partially polymerized hydrogels were removed from PDMS stamps and cured with feature side up for one additional minute. PEG-DA ($Mw = 6$ kDa)–micropatterned gels were made in the same manner with 0.2% Irgacure 2959. The resulting polymerized PEG–fibrinogen or PEG-DA micropatterns were hydrated in PBS and UV sterilized before cell seeding. Measurements of PEG–fibrinogen feature sizes done after hydration in PBS indicate that microwells were 522 ± 27 μm in diameter and 213 ± 19 μm deep, measured by confocal imaging of microwells filled with 20-μm-diameter fluorescent beads (Fig. 1).

Evaluation of mechanical properties of PEG–fibrinogen hydrogels

Polymer solutions that matched the composition of those used in cellular experiments, of approximately 250 μL volume, were polymerized between 18 mm coverslips. The bottom coverslip was functionalized with aminopropyltriethoxysilane to covalently link the hydrogel for ensuing testing. Several samples from four different PEG–fibrinogen stocks were measured using atomic force microscopy-enabled nanoindentation. An atomic force microscope (MFP-3D; Asylum Research) was used for all mechanical characterization experiments conducted on hydrogels in 1×PBS at room temperature. Calibration of atomic force microscope cantilevers of spring constant $k = 34.60$ pN/nm and nominal probe radius $R = 25$ nm (MLCT; Veeco) was conducted as previously described.[42,43]

For each measurement of Young's elastic moduli, a grid of 16×16 (256 total) indentations to maximum depths of 25 nm were acquired over a 50 μm^2 area on each hydrogel to account for material inhomogeniety. Acquired probe deflection-displacement responses were converted offline (Igor Pro; Wavemetrics), to force-depth responses. Young's elastic moduli E were calculated by applying a modified Hertzian model of spherical contact to the loading segment of the force-depth response, as detailed elsewhere,[42,43] with the scientific computing software MATLAB (2007a, TheMathWorks). The computed elastic moduli E are reported as average ± standard error of measurement. The elastic modulus of the gels comprising 5% exogenous PEG diacrylate was 17.5 ± 0.3 kPa (mean ± standard error of the mean), which is intermediate to that of estimates for normal and diseased liver as discussed in the text.[33–35]

Immunofluorescence microscopy

Samples were fixed in 3.7% formaldehyde in PBS for 25 min at room temperature, permeabilized with 0.1% Triton-X for 10 min at 4°C, and washed twice with PBS for 15 min each. For accumulation of growth factors, samples were blocked for 1 h with 10% normal goat serum (Invitrogen) and 1% bovine serum albumin (Sigma). Samples were incubated with primary antibodies overnight at 4°C and then washed thrice with PBS for 20 min each. Samples were then incubated with secondary antibodies, AlexaFluor568 or AlexaFluor488 phalloidin, and 4′,6-diamidino-2-phenylindole (DAPI) or Hoechst nuclear stains for 1 h at room temperature with protection from light before being washed thrice with PBS for 20 min each. Samples were then stored in PBS at 4°C with protection from light. Visualization was done using a Zeiss Observer A1 microscope equipped with a Photometrics Quant EM S12SC camera and BD CARV II spinning disc confocal (Biovision Technologies). Images were acquired using MetaMorph software. Confocal images shown in the figures in this report are single confocal cross-sections taken within the centermost 25% of the tissue structure. Tissue structures were typically 180–200 μm deep in the absence of inhibitors (and comparable for presence or absence of soluble EGF), 120–150 μm deep (when intact) in the presence of cRGD integrin inhibitor, and 100–120 μm deep in the presence of mAb225 EGFR inhibitor. Zeiss STEREO Discovery V12 stereoscope equipped with AxioCam and fluorescent lamp was used for observing accumulation of TGF-α, EGF, and HGF in PEG–fibrinogen microwells. Consistent exposure times were maintained to assess relative fluorescent staining between conditions. For EGF, TGF-α, and HGF immunofluorescence detection, samples incubated with secondary antibody alone were imaged to ensure that the observed fluorescence was not due to an artifact, such as nonspecific secondary antibody binding or autofluorescence.

For visualization of functional bile canalicular networks, samples were washed thrice with Hanks' balanced salt solution and then incubated for 10 min at 37°C with 2 μM 5(and 6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDFDA; Invitrogen) and 16.2 μM Hoechst stain. After incubation, confocal images were taken at days 3 and 6 (representative image from day 6 is depicted in Fig. 5E).

Primary antibodies include anti-rat FN at 1:100 (Millipore), anti-rat CD26 at 1:100 (BD Pharmingen), anti-rat TGF-α at 1:500 (Abcam), anti-rat HGF at 1:200 (Abcam), and anti-rat EGF at 1:200 (Abcam). Secondary antibodies at 1:200 and phalloidin dyes at 1:200 were all AlexaFluor conjugates (Invitrogen). Nuclear stains were used at 1 μg/mL for DAPI (Sigma) and 16.2 μM for Hoechst 33342 (Invitrogen).

Quantification of cell viability

Cell viability was determined using the Live/Dead Viability/Cytotoxicity Kit (Invitrogen). Cells were cultured as described above on PEG–fibrinogen gels for the indicated time periods, with or without 10 ng/mL EGF, or in the presence of 10 μM cRGD or 10 μM mAb225, or cultured on adsorbed collagen I in the presence of 10 ng/mL EGF. For PEG–fibrinogen gels, confocal Z-stack images of three to five randomly chosen microwells were taken from each of three to four gel samples per time point using a 10× objective to capture the entire well (10 μm step size in z-stacks). Total cell number was determined by DAPI staining of nuclei and the number of nonviable cells was determined by ethidium bromide nuclear staining. A total of 200–600 nuclei were counted in each microwell to obtain total cell number. For adsorbed collagen samples, in each well, four to six randomly chosen

fields (one from each quadrant of the well) were observed using a 10× objective, and all nuclei were counted in each field (300–600 total nuclei per field). The experiments were performed at least three times (for both PEG–fibrinogen and adsorbed collagen samples) and data were statistically analyzed with analysis of variance followed with Tukey's test with alpha = 0.05.

Quantification of albumin and urea production

Culture medium was replaced at 48 h prior to collection on the indicated days. Albumin levels were determined using Rat Albumin ELISA Kit (Bethyl Labs). Urea levels were determined using the QuantiChrom Urea Assay Kit (BioAssay Systems). Samples, standards, and controls were tested in duplicates and experiments were repeated three to six times. Data were normalized to account for sample volumes for both assays. Results are reported as nanograms of product (urea or albumin) per milliliter per day (Fig. 7). The data were normalized to account for cell number at day of sample collection and are presented as micrograms of product (urea or albumin) per 10^6 cells per day (Supplementary Fig. S5). Cell number per day was calculated based on the total cell counts from the cell viability data for each specific time point. The data were statistically analyzed with analysis of variance followed with Tukey's test with alpha = 0.05.

Results

Micromolding of PEG–fibrinogen gels to create a 3D niche for primary hepatocytes

A PEG–fibrinogen hydrogel formulation was chosen to create niches for primary hepatocytes based on several criteria: amenable to micromolding (to isolate small numbers of hepatocytes); intrinsically nonadhesive for hepatocytes, but possessing specific adhesive domains for cell-secreted matrix, to allow interpretation of effects from retention of cell-secreted matrix; physiologically relevant stiffness (similar to liver) should cellular ECM secretion result in adhesion of the cells with the microwell walls. The precise permeability properties of the hydrogel were a secondary consideration, as the cell aggregate itself provides a means of concentrating autocrine factors locally during initial stages of culture, compared with the case of 2D minimally adhesive surfaces. These criteria were not met by commonly used hydrogels such as collagen and agarose. PEG–fibrinogen hydrogels met these criteria while providing the additional advantage that secreted FN can interact with fibrinogen subunits within the hydrogel, improving sequestration of cell-derived matrix while only providing adhesion upon autocrine FN retention.

The micromolding scheme is presented in Figure 1, wherein arrays of microwells filled with 20 μm fluorescent beads illustrate the appearance of the wells following final processing steps. The dimensions of the cylindrical features (nominal height, $h = 200$ μm, and nominal diameter, $d = 500$ μm; actual $h = 213 \pm 19$ μm, $d = 522 \pm 27$ μm) were guided by previous studies on the length scales for self-assembly of isolated hepatocytes (confirmed in these studies; data not shown) and diffusion into 3D cultures.[44] Bulk elastic moduli of these micromolded hydrogels were measured via atomic force microscopy-enabled indentation ($E = 17.5 \pm 0.3$ kPa [mean ± standard error of the mean]), which agrees reasonably well with that reported for liver tissue.[33–35] PEG–fibrinogen hydrogels can be proteolytically degraded, with degradation rates highly dependent on the content of additional PEG-DA added during polymerization.[45] The formulation used here, containing 5% PEG-DA, is in a regime associated with relatively slow degradation, and we saw no evidence of bulk degradation during the course of the experiments.

Primary hepatocytes seeded into microwells in the absence of exogenous EGFR ligands or adhesive matrix molecules aggregated into tissue-like structures over the first 24 h, formed strong adhesions to the gel matrix, and persisted in tissue-like structures over 10 days of culture (Fig. 1B). A modest but noticeable compaction of the tissue structures occurred in the first few days of culture, as expected by increasing cell–cell contacts causing the structures to pull away from some regions of the walls of the microwells, while remaining firmly attached over about 50% of the contact area. Attempts to culture primary hepatocytes on 2D (nonmolded) PEG–fibrinogen gels were unsuccessful; cells did not attach to the gel, instead forming floating spheroids with little retention of FN, in keeping with previous attempts to culture hepatocytes on fibrinogen.[17] Similar results were found with micromolded PEG-DA (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/tea) and agarose gels (data not shown), on which, despite intact features, cells did not attach or persist in microwells but rather formed viable floating spheroids.

Primary hepatocytes retain cell-derived FN and growth factors within micropatterned wells

Hepatocytes lack receptors for fibrinogen. Hence, the observation that primary hepatocytes formed adherent tissue-like structures in the wells of PEG–fibrinogen gels in the absence of serum or other sources of exogenous ECM or growth factors suggested that the microwells were capable of retaining autocrine factors, including ECM, necessary for cell survival. To determine if primary hepatocytes were able to retain FN matrix and growth factors produced by cells post-isolation, immunofluorescence microscopy was used to monitor the presence of several key autocrine factors produced by hepatocytes.

FN—ubiquitously present in liver ECM—is assembled from its soluble form into fibrils in an integrin-dependent manner ($\alpha5\beta1$ in the case of hepatocytes).[46] In culture, thin (5–25 nm) fibrils forming interconnected networks appear in the extracellular environment early in culture, form networks around cells, and mature into thicker fibril bundles at later stages of culture.[46] Immunofluorescence staining, as illustrated by confocal imaging of a single slice in the central tissue region, shows the presence of FN in and around primary hepatocytes seeded and cultured in micromolded PEG–fibrinogen hydrogels, with a staining pattern that suggests formation of fibrils and their maturation over time into fibril bundles (Fig. 2). Hepatocytes were cultured, as described earlier, for 3, 7, and 10 days before being fixed, stained with antibodies against FN, and imaged by confocal microscopy to view the region of tissue approximately halfway between the top and bottom of the tissue structure (75–100 μm below the top of the gel). Culture medium was serum free and did not contain any exogenous matrix molecules or peptide growth

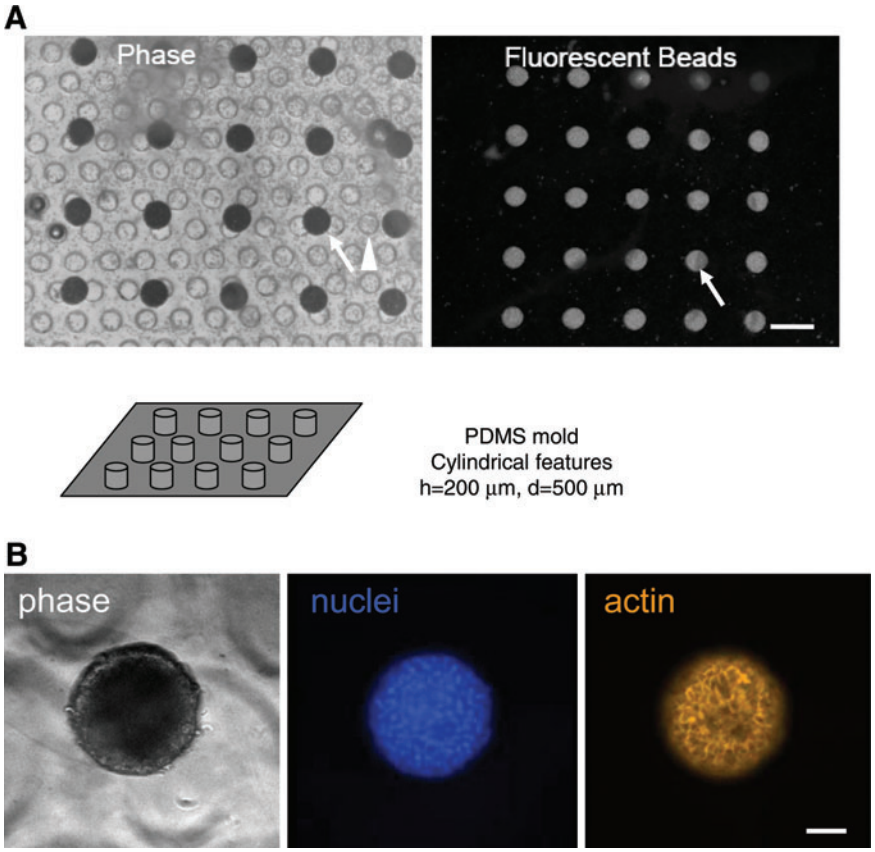

FIG. 1. Micromolding of PEG–fibrinogen hydrogels. (A) PEG–fibrinogen gels were polymerized as described in the Materials and Methods section, on top of a polycarbonate support scaffold visible by phase microscopy. After polymerization, fluorescent microbeads were placed on the patterned gel and sonicated for 30 s to allow the beads to settle inside the negative features of the PEG–fibrinogen gel. The gel was subsequently washed with phosphate-buffered saline to remove floating beads and imaged by fluorescence microscopy. Microwells filled with beads are indicated by arrow; support scaffold channels are indicated by arrowhead. For reference, the microbeads have $d = 20\,\mu m$ and support scaffold channels have $d = 340\,\mu m$. Scale bar = 1 mm. **(B)** Freshly isolated primary hepatocytes were plated onto molded gels, as described in Materials and Methods, at a density of 100,000 cells/cm^2 (720,000 cells/mL) in hepatocyte growth medium lacking growth factors (except for insulin). Three days after plating, cultures were fixed and stained to label the nuclei (blue) and the actin cytoskeleton (orange). Single confocal cross-sections taken within the centermost 25% of the structure are shown. Scale bar = 100 μm. PEG, polyethylene glycol. Color images available online at www.liebertonline.com/tea.

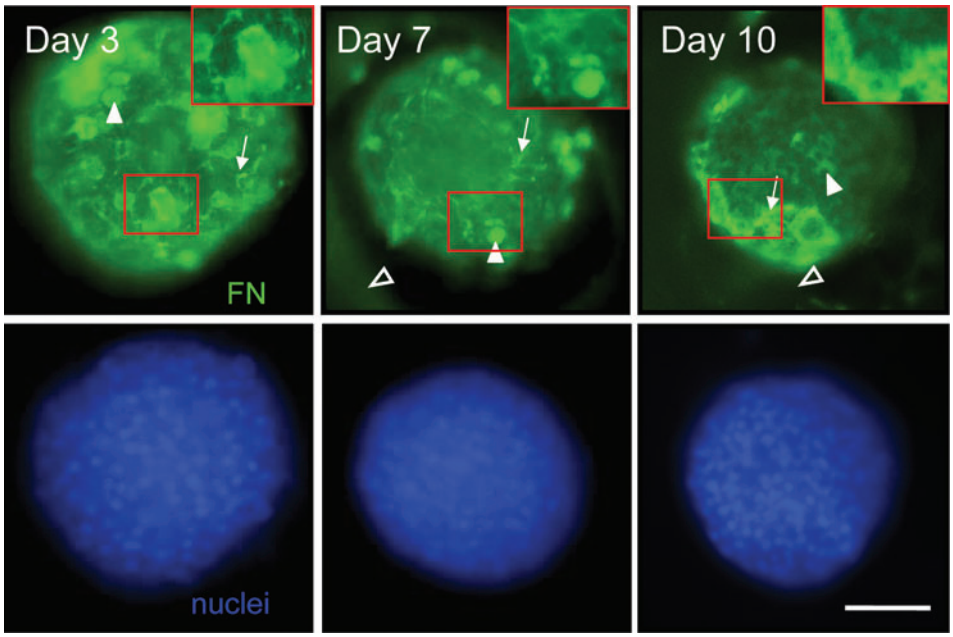

FIG. 2. Primary hepatocytes retain FN matrix within micropatterned wells. Primary hepatocytes were plated as previously described. At 3, 7, and 10 days, cells were fixed and stained for FN (green) and nuclei (blue). Confocal images were taken at 20×and digital zoom of red highlighted regions is illustrated in top right insets. Single confocal cross-sections taken within the centermost 25% of the structure are depicted. Note intracellular FN (arrowheads), FN fibrils (arrows), and staining for secreted, soluble FN trapped in the walls of the microwell (open arrowheads). For phase images, see Figs. 1 and 6 (day 3), Supplementary Fig. S4 (day 7), and Supplementary Fig. S5 (day 10). Scale bar 100 μm. FN, fibronectin. Color images available online at www.liebertonline.com/tea.

factors, except for insulin; hence, observed FN was produced by hepatocytes. FN with a bright staining pattern characteristic of fibrils is present surrounding cells in the central region of the tissue structures by day 3 and persists at days 7 and 10 (Fig. 2, arrows), with a coarsening and thickening of the apparent network by day 10 (Fig. 2). In addition to fibrils, diffuse staining is observed within the gel adjacent to the microwell by day 7 (Fig. 2). The diffuse appearance of FN staining inside the gel indicates that soluble FN secreted by hepatocytes diffuses into the gel and remains associated with it, as anticipated by the known propensity of FN to associate with regions of fibrinogen. We postulate that interactions between FN and fibrinogen in the gel provide a bridge to link hepatocyte tissue structures firmly to the gel, even as the tissue structures appear to contract slightly and draw away from some regions of the microwell wall at later stages of culture (Fig. 2). The gap between the tissue structure and the gel following this slight contraction appears as a dark region between the brightly staining tissue and the diffuse-staining support gel. The retention of soluble FN within the microwell support structure and the assembly of FN fibrils within the tissue aggregate are unique to cultures within PEG–fibrinogen microwells, as cells cultured in PEGDA microwells show diminished fibril assembly within the tissue aggregate and no diffuse staining in the gel wall adjacent to the tissue structure (Supplementary Fig. S1).

The requirement for growth factor signaling, including HGF and EGFR ligands (including EGF and TGF-α), to maintain primary hepatocyte cultures has been well documented.[47–50] The ability of primary hepatocytes to form tissue-like structures on micromolded PEG–fibrinogen gels in the absence of any of these growth factors provided exogenously implicates autocrine signaling. To examine the presence of autocrine growth factors, primary hepatocytes were cultured in serum-free medium devoid of exogenous growth factors and then fixed and stained with antibodies against EGF, HGF, and TGF-α at days 3, 7, and 10. This protocol detects membrane-bound proforms of the growth factors as well as shed factors that become cross-linked to ECM or cell surfaces during fixation. Tissue structures were imaged with low magnification to assess uniformity of stains across multiple wells. All three growth factors are present in the day 3 tissue structures formed by culture of primary adult rat hepatocytes in the microwells (Fig. 3) and expression is sustained through days 7 and 10 (Supplementary Fig. S2). The apparent lack of staining in the gel adjacent to the tissue structure is not surprising, as growth factors are likely consumed locally in autocrine fashion such that only low (undetectable) concentrations escape into the gel.

Autocrine matrix and growth factors are necessary and sufficient to maintain the viability of primary hepatocytes in culture

The formation and maintenance of tissue-like aggregates of primary hepatocytes within microwells of nonadhesive PEG–fibrinogen was associated with retention of autocrine factors including FN (Fig. 2), EGF, TGF-α, and HGF (Fig. 3). Previous studies in 2D culture have shown that survival of primary adult rat hepatocytes is diminished but not abolished in the absence of EGFR ligands.[51–53] To determine if autocrine signaling from cell-secreted FN and EGFR ligands is sufficient for cell survival the number and viability of primary adult rat hepatocytes cultured on the PEG–fibrinogen gels in the absence of serum or exogenous growth factors was measured as a function of time in culture. Total cell numbers were enumerated by counting all nuclei in 3–5 microwells in 3–4 gel samples (at least 200–600 nuclei were counted in each microwell) and nonviable cells were labeled by uptake of ethidium bromide. In micromolded gel substrates, cell number showed a slight (~7%) but statistically insignificant decline over the 10 days of culture, with no statistically significant difference between cultures with or without soluble EGF (Fig.

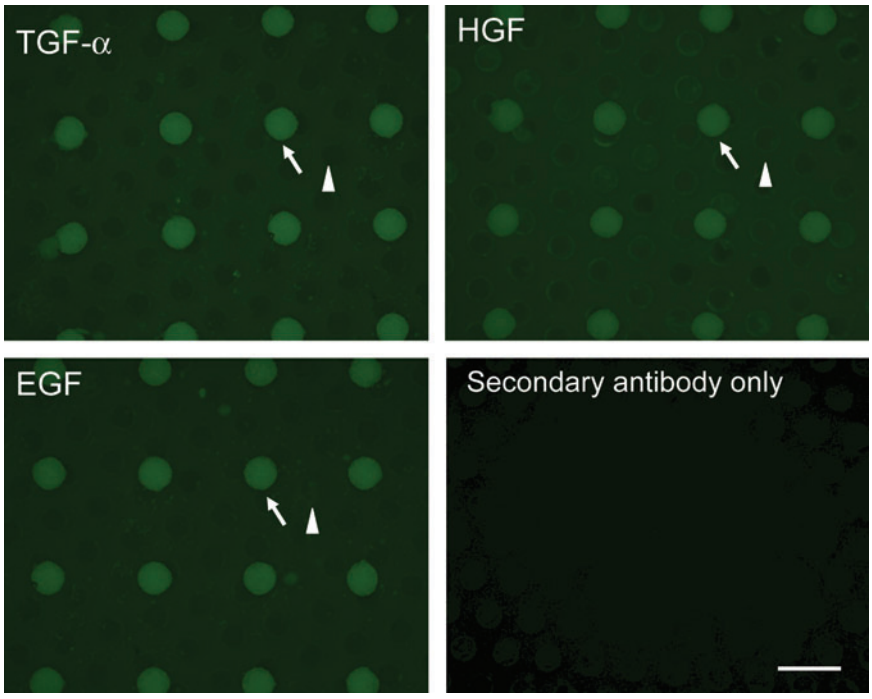

FIG. 3. Primary hepatocytes retain autocrine growth factors in micropatterned wells. Cells were cultured as previously described, in the absence of serum or exogenous growth factors (except for insulin). After 3 days, samples were fixed and stained with antibodies against EGF, HGF, and TGF-α. As a negative control, a sample was incubated with secondary antibody alone to confirm that signal was from primary antibody. Fluorescence indicates retention of TGF-α, EGF, and HGF. Microwells filled with cells are indicated by arrow; support scaffold channels are indicated by arrowhead. Support scaffold channels have $d = 340\,\mu m$. Scale bar = 1 mm (magnification: 35×). For days 7 and 10, see Supplementary Figure S2. EGF, epidermal growth factor; HGF, hepatocyte growth factor; TGF, transforming growth factor. Color images available online at www.liebertonline.com/tea.

4A). On 2D collagen I-coated substrates in the presence of EGF, a modest (~10%) but statistically significant increase in cell number was observed over 10 days in culture (Fig. 4A) consistent with other reports for these culture conditions.[29] A similar trend was observed in cell viability during the culture period. Cell viability was assessed by the fraction of cells that excluded ethidium bromide versus total cells stained with DAPI at days 1, 3, 7, and 10. Cells plated on adsorbed collagen I supplemented with EGF exhibit a high level of viability (Fig. 4B). Viability of cells in PEG–fibrinogen cultures was 80%–90%, with no statistical difference between cultures supplemented with 10 ng/mL EGF and those without. The fate of dead cells was not assessed, and it is possible that cells appearing as nonviable at early time points persist in culture. These data indicate that the autocrine factors present within the microwells are sufficient to sustain hepatocyte viability in long-term culture.

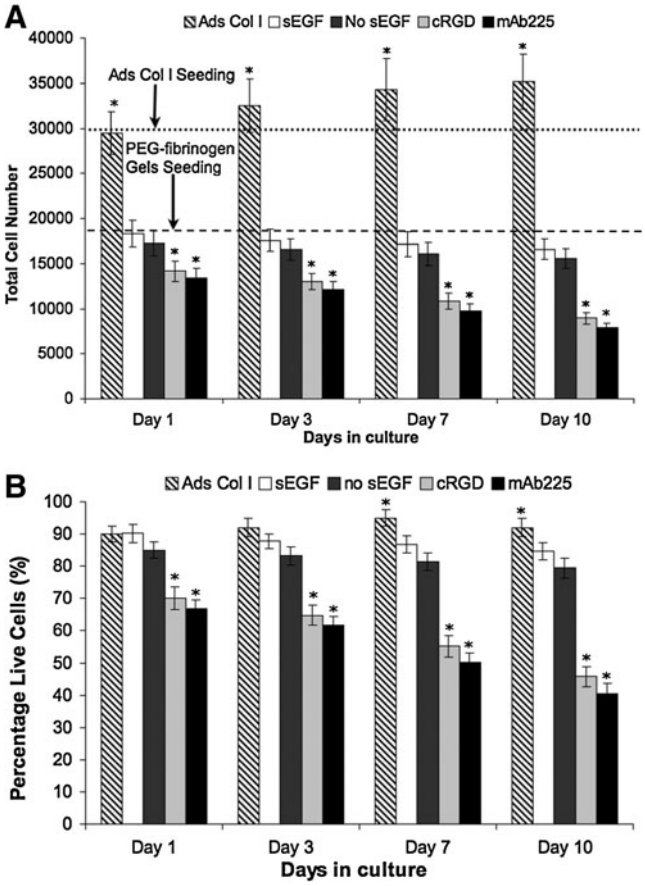

FIG. 4. Autocrine signaling is necessary and sufficient for the survival of primary hepatocytes *in vitro*. (A) Total cell numbers as a function of time in culture. **(B)** Quantification of cell viability expressed as percentage of viable cells. Freshly isolated primary hepatocytes were plated onto molded gels at a density of 100,000 cells/cm^2 in hepatocyte growth medium with 10 ng/mL EGF (white, sEGF), no EGF (dark gray, no EGF), no EGF with 10 μM cRGD peptide (light gray, cRGD), or no EGF with 10 μM mAb225 (black, mAb225). Hepatocytes were cultured on adsorbed collagen I at a density of 100,000 cells/cm^2 in hepatocyte growth medium supplemented with 10 ng/mL EGF (diagonal pattern, Ads Col I). At the indicated time points, viable cells were identified by their ability to exclude ethidium bromide and total cell numbers were determined with 4′,6-diamidino-2-phenylindole staining. The horizontal lines depict total number of cells seeded on PEG–fibrinogen gels and adsorbed collagen substrates. For PEG–fibrinogen gels, total number of cells effectively seeded into 35 microwells per culture is based on measurements of the number of nuclei in 20 microwells in four samples at 24 h after plating. *Statistical significance when compared with PEG gel-soluble EGF condition on that specific day; $p < 0.05$, $n > 3$. cRGD, cyclic arginine–glycine–aspartate.

To determine if signaling from either integrin α5β1 or EGFR is necessary for hepatocyte survival, primary hepatocytes were cultured as described above but in the presence of inhibitors. To block α5β1-mediated signaling, cells were cultured with a cRGD peptide to prevent FN and other ECM ligands from binding to this integrin. To block EGFR signaling, cells were cultured in the presence of a monoclonal antibody against EGFR (mAb225), which blocks ligand binding to EGFR, inhibiting autocrine signaling by EGF, TGF-α, and any additional autocrine EGFR ligands such as amphiregulin and HB-EGF that may be present. mAb225 binding induces slow internalization and trafficking of EGFR, but does not result in phosphorylation and activation of the EGFR or any known EGFR-mediated signaling events.[54] mAb225 was replenished at each medium change to mitigate effects of receptor-mediated downregulation. The presence of either inhibitor resulted in comparable declines in cell number (Fig. 4A) and cell viability (Fig. 4B) compared with control cultures. Total cell numbers in the presence of inhibitors on day 1 were ~85% of control values and declined further to ~50% of control values by day 10 (Fig. 4A), a trend mirrored by cell viability (Fig. 4B). Both inhibitors (i.e., cRGD or EGFR function-blocking antibody) caused a marked decrease in the levels of cell-associated EGF, TGF-α, and HGF as observed by immunofluorescent staining of microwell structures (Supplementary Fig. S3). Compared with control cultures, a faint background staining was observed uniformly on the gels for the EGFR-inhibited case, suggesting that cells are still shedding these factors, and in the absence of EGFR uptake, they are adsorbing to the gel-associated ECM. The apparent loss of tissue-associated staining for growth factors in the inhibited cases may be attributed to the decline in cell number due to decreased viability or due to cross-talk between integrins and growth factors controlling a positive feedback loop of autocrine production, an area for further study.

Micropatterned PEG–fibrinogen hydrogels support formation of functional bile canalicular networks

In vivo, hepatocytes adopt an atypical cell polarity in which the apical cell surface composes the bile canalicular network into which bile is secreted and funneled to the bile duct. To determine the presence of bile canaliculi, cells cultured for 3 days were stained with antibodies to CD26, a marker of the apical surface, and imaged by confocal microscopy. Figure 5A and B show a cross-sectional image of a representative tissue-like structure with apical staining at the cell–cell junctions where canaliculi form. Another aspect of this polarity is the presence of FN at the apical surface: unlike other epithelia, hepatocytes lack a canonical basement membrane; instead, FN is found at apical, basal, and lateral surfaces.[55] To determine whether FN was localizing to the apical surface in addition to the general staining seen in Figure 2, cultures were

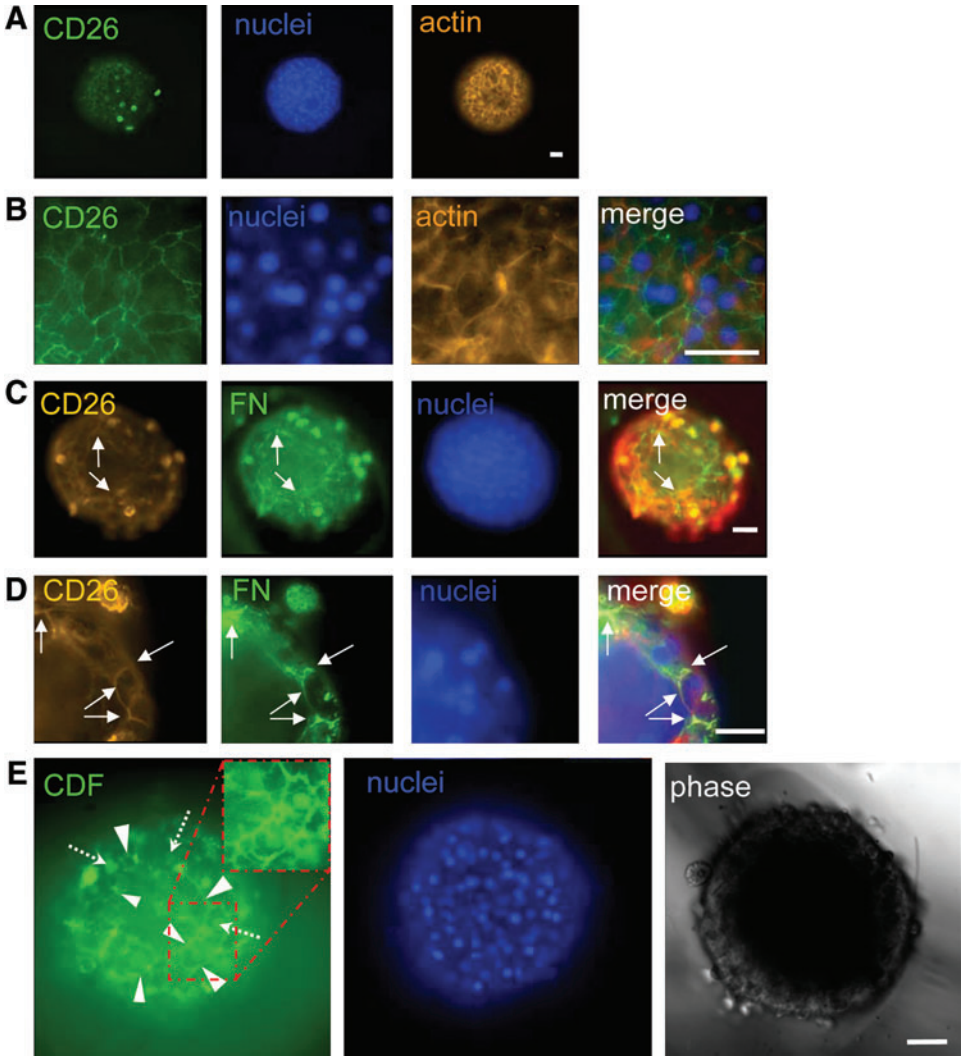

FIG. 5. Micropatterned PEG–fibrinogen hydrogels support three-dimensional polarization of primary hepatocytes and the formation of a functional bile canalicular network. Cells were plated and cultured in hepatocyte growth medium without EGF as previously described and then fixed and stained for the indicated epitopes. Single confocal cross-sections taken within the centermost 25% of the structure are depicted. **(A, B)** Cells cultured for 3 days and stained for CD26 (green), nuclei (blue), and the actin cytoskeleton (orange); samples are imaged at 10×**(A)** and 63×**(B)**. **(C, D)** Cells cultured for 7 days and stained for CD26 (orange), FN (green), and nuclei (blue); samples are imaged at 20×**(C)** and 40×**(D)**. **(E)** Visualization of functional bile canaliculi by confocal microscopy on day 6 using 5(and 6)-carboxy-2′,7′-dichlorofluorescein (CDF) diacetate at 20×(with digital zoom of red highlighted region in top right inset). For reference, support scaffold channels have $d = 340\,\mu m$. Scale bar = 50 μm in each image. Note regions of plasma membrane showing CD26 and FN colocalization (arrows), and concentrated CDF staining in the three-dimensional tissue structure, which is similar to CD26 staining in **A** and **B** (arrowheads = bile canaliculi; dotted arrows = intracellular uptake of CDF). Color images available online at www.liebertonline.com/tea.

costained with antibodies against FN and CD26. Figure 5C and D show that FN does colocalize with CD26 at discrete regions of the apical domain of the hepatocytes. The functionality of the canalicular network was examined using CDFDA. CDFDA is actively taken up by hepatocytes, deesterified intracellularly to fluorescent CDF, and secreted into the canaliculi, allowing visualization of canalicular domains with intact tight junctions during a 10–15 min period before contraction of canaliculi releases their contents into the medium. Results of this assay are shown in Figure 5E, wherein the CDF is visible in a pattern similar to the CD26 staining. Thus, primary hepatocytes are capable of recapitulating aspects of their complex *in vivo* morphology *in vitro* with only autocrine signals to direct them.

Autocrine FN is necessary for formation and maintenance of tissue-like structures

FN is abundant in the liver sinusoid where this ECM protein is in direct contact with the hepatocytes that are responsible for production of plasma FN. Similarly, in micromolded PEG–fibrinogen gels, primary hepatocytes retain FN and create fibrillar networks in the extracellular environment. To determine whether this autocrine ECM is crucial for the survival of primary hepatocytes and the formation or maintenance of tissue-like structures, hepatocytes were cultured in the presence of a cRGD peptide that blocks cell-surface α5β1 integrins from binding FN. Cells were cultured as described above but 10 μM of the cRGD peptide inhibitor was included at the time of plating and subsequent media changes. Cultures were fixed at 3, 7, and 10 days and stained with anti-FN and anti-CD26 antibodies.

Blocking α5β1 integrin prevents the formation of viable tissue-like aggregates such as those seen in control cultures on day 3 and, instead, results in dissociated, rounded cells (Fig. 6) that gradually become more diffuse by days 7 and 10 (Supplementary Figs. S4 and S5), concomitant with significantly reduced viability compared with controls (Fig. 4). In control cultures, abundant fibrillar FN networks are observed in the microwells by day 3 (Figs. 5 and 6, top), whereas cultures with the cRGD inhibitor lack detectable extracellular FN, even though intracellular FN is present. Further, cells cultured in

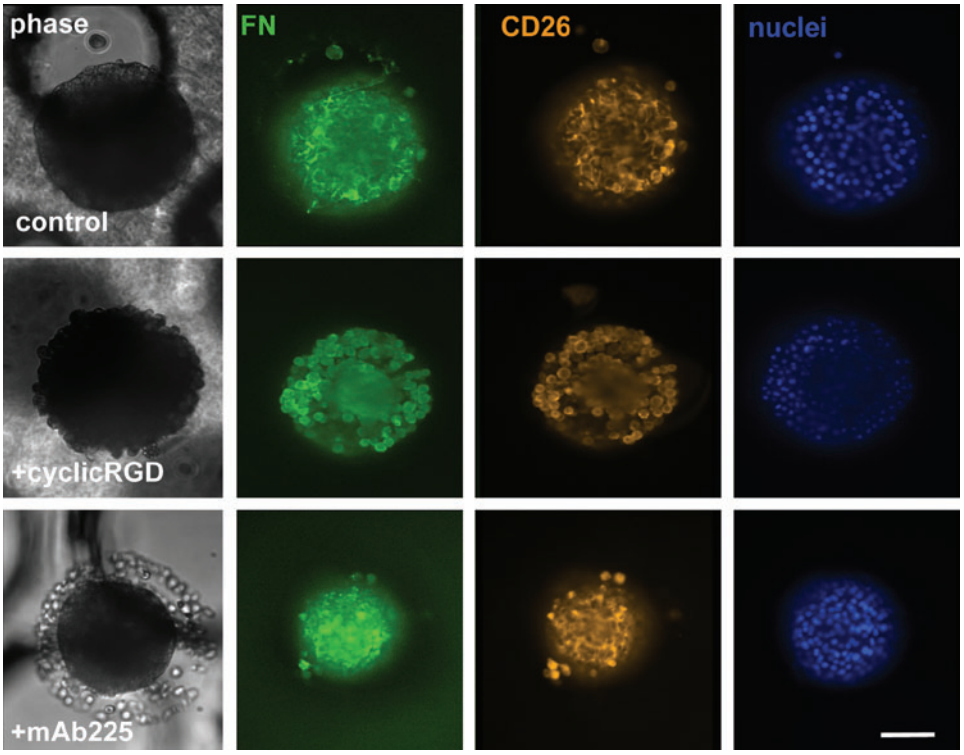

FIG. 6. Blocking α5β1 but not EGFR disrupts tissue-like structures. Cells were plated as described above and cultured for 3 days in the absence or presence of 10 μM cRGD peptide or mAb225. After 3 days, cultures were fixed and stained for FN (green), CD26 (orange), and nuclei (blue). Note the absence of FN fibrils or discrete CD26 staining and the loose, dissociated cells in the microwells of cultures with cRGD. Also note the smooth edges of the aggregates, loose cells in the bottom of the well, and staining for FN and CD26 similar to control culture in the microwells of cultures with mAb225. For reference, support scaffold channels have $d = 340$ μm. Scale bar = 100 μm. For days 7 and 10, see Supplementary Figures S4 and S5. EGFR, epidermal growth factor receptor. Color images available online at www.liebertonline.com/tea.

the presence of cRGD fail to organize CD26 into canalicular like-structures at early (Fig. 6) or late (Supplementary Figs. S4 and S5) stages of culture, instead showing diffuse staining throughout the cell body. The rounded, individual cell morphologies revealed by both FN and CD26 staining show that the cells treated with cRGD have little functional cell–cell contact compared with control cultures. This combined failure to localize CD26 and the absence of extracellular FN fibrils is in stark contrast to images of control tissue-like aggregates, which show a consistent colocalization of FN fibrils with bile canalicular structures (Fig. 5C, D).

To determine if EGFR signaling was also crucial to the formation or maintenance of the tissue-like structures, hepatocytes were cultured in the presence of the function-blocking EGFR antibody mAb225. The antibody was added to cultures at the time of plating and subsequent media changes, and then cells were fixed and stained for FN and CD26 at 3, 7, and 10 days. The tissue structures formed under inhibition of EGFR had a much more compact morphology than controls at day 3, with many dead, dissociated cells in the bottom of the microwell (Fig. 6, compare phase and DAPI stains). This highly compact morphology compared with controls (Fig. 6) arose from both reduction in cell number by about 40% compared with controls (Fig. 4) together with a closer spacing of cells than in the RGD-inhibited case (where cells were partially or completely dissociated), as evidenced by the close location of nuclei in EGFR-inhibited cultures; these patterns were accentuated by day 7 (Supplementary Fig. S4) and again by day 10 (Supplementary Fig. S5). Interestingly, the total cell numbers and viabilities as a function of time were comparable for both the integrin-inhibited and EGFR-inhibited cultures (Fig. 4), yet these cultures had strongly divergent morphologies, with RGD-containing cultures lacking a tissue-like structure (Fig. 6 and Supplementary Figs. S4 and S5). The pattern of FN and CD26 staining in EGFR-inhibited cultures appears to be predominantly diffuse in intracellular (FN) and membrane (CD26) staining (Fig. 6 and Supplementary Figs. S4 and S5), without the reticular structures seen in controls; bright, condensed regions of staining appear related to the compactness of the culture rather than functional cell–cell contacts, although occasional CD26 reticular structures are seen in the EGFR-inhibited case.

PEG–fibrinogen hydrogels promote maintenance of hepatocyte metabolic functions in vitro

Primary hepatocytes rapidly dedifferentiate and lose metabolic functions when grown by standard cell culture techniques, as determined by measuring certain standard metabolic functions of hepatocytes: conversion of ammonia to urea and the production of albumin. As the PEG–fibrinogen gels are capable of recapitulating other aspects of hepatocyte function, such as formation of bile canaliculi and production of FN, their metabolic function was monitored and compared with hepatocytes cultured on tissue culture plastic with adsorbed collagen I. Cells were cultured as described above for 10 days and the production of albumin and urea in the conditioned media was assessed. A comparison of the total daily amount of albumin secreted by cells maintained under various culture conditions reveals that at all time points measured, hepatocytes cultured in the micromolded PEG–fibrinogen gel format produce statistically greater amounts of albumin compared with cells on adsorbed collagen I (Fig. 7A). Interestingly, minimal to no difference is observed in cultures maintained with or without EGF. The presence of inhibitors of integrin α5β1 or EGFR function suppress albumin secretion dramatically to levels

<20% of control levels. These trends are further accentuated when albumin production rates (shown in Supplementary Fig. S6A) are normalized to viable cell number (Fig. 7A). Production of urea follows similar trends across treatment conditions (Fig. 7B and Supplementary Fig. S6B). These results are in keeping with results presented elsewhere in this report that the retention of autocrine FN and growth factors allow hepatocytes *in vitro* to maintain aspects of their *in vivo* morphology and function.

Discussion

The data presented here implicate autocrine ligands of α5β1 and EGFR as effectors of hepatocyte function in long-term culture by combining immunostaining, illustrating the presence of a subset of known ligands for each receptor type (FN for integrin α5β1 and TGF-α and EGF for EGFR) with function-blocking inhibitors. In the absence of exogenous adhesion ligands and growth factors, primary hepatocytes cultured in micromolded PEG–fibrinogen gels are capable of modifying their microenvironment to maintain their differentiation and metabolic function. The PEG–fibrinogen hydrogel system offered a practical advantage for microwell culture of hepatocytes, in that it is not intrinsically adhesive to hepatocytes, which are not known to express receptors for fibrinogen, but can become adhesive in the presence of cell-secreted ECM.

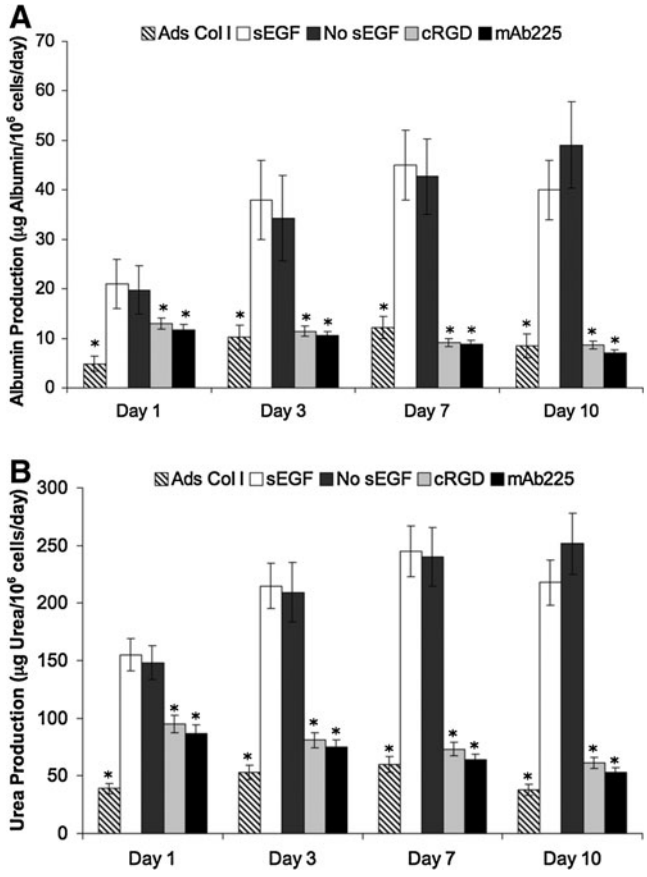

FIG. 7. PEG–fibrinogen hydrogels promote maintenance of hepatocyte metabolic functions *in vitro*. Primary hepatocytes were cultured on micromolded PEG–fibrinogen gels or adsorbed collagen I as described in Materials and Methods. Conditioned medium was collected at the indicated time points and metabolites of interest were quantified as described in the Materials and Methods section. Samples, standards, and controls were tested in duplicate. **(A)** Albumin synthesis; **(B)** urea synthesis. *Statistically significant difference from sEGF-supplemented PEG–fibrinogen samples at a specific day; $p < 0.05$, $n > 3$. Data has been normalized to number of viable cells at each day. For absolute (non-normalized) rates of secretion, see Supplementary Figure S6.

In comparing behavior of hepatocytes seeded onto 2D PEG–fibrinogen substrates formed by the same gelation process to behavior of cells in microwells, we found that hepatocytes adhered to the gels only when cultured in microwell format. Although hepatocytes did form spheroidal aggregates on the 2D substrates, as they have been observed to do on many minimally adhesive 2D substrates,[12,45] these aggregates failed to adhere to the 2D PEG–fibrinogen gel substrate. In the microwell format, the local cell environment fosters accumulation of cell-secreted factors simply because of very high local cell density[22] compared with 2D. This phenomenon may be further accentuated if the gel also serves as a diffusion barrier or if it binds factors to provide a local depot for sequestration. The precise permeability properties of these gels were not measured; however, they are expected to be less permeable than PEG–fibrinogen formulations commonly used for cell encapsulation purposes[36,39,40] as they were formed with a relatively high ratio of 6 kDa PEG-DA (5%) to PEG–fibrinogen (3.6%). Pure PEG-DA gels formed with 10–20 kDa PEG with 10% polymer content present significant diffusion hindrance to proteins above 20 kDa, including HGF, which has a molecular weight ~60 kDa[56,57]; small proteins such as EGF likely diffuse relatively unhindered in the as-polymerized gels.

Thus, the differences in formation of adherent cell aggregates during the first day of culture in the microwell format compared with 2D culture on the same substrate may arise from higher local concentration of small peptide factors such as EGF, because of locally high cell concentrations in microwells compared with 2D. These effects may be coupled with enhanced retention of larger autocrine factors such as HGF and enhanced local concentrations of FN due to reduced permeability of the gel to large proteins. Another factor that may be operative in early stages to facilitate interactions between ECM and the gel in microwell format compared with 2D is attainment of locally high concentrations of proteases. Extracellular proteases may degrade the gel to increase the surface area for gel–ECM interaction in the microwell compared with 2D format. Secreted proteases range in size from about 20 kDa to over 100 kDa; hence, a substantial fraction of proteases would likely exhibit hindered diffusion in the gel compared with culture medium. As reported previously,[36] PEG–fibrinogen gels polymerized with high additional PEG-DA macromer content exhibit relatively slow enzymatic degradation compared with pure PEG–fibrinogen gels, but even a modest degree of local remodeling may enhance adhesion of ECM and cells. Although other types of cells encapsulated in or cultured on PEG–fibrinogen gels have been observed to migrate into gels, we did not observe cellular ingrowth into the gels or observe any signs of bulk gel degradation (e.g., swelling or fracturing of the gels) for cells cultured in the microwell format. Adult hepatocytes are not highly migratory[13] and proteases tend to act in a highly local fashion; hence, we would not anticipate,

nor did we observe, bulk gel degradation for a format where cells are so highly localized.

Previous work has found a role for FN in a variety of developmental, regenerative, and disease processes in the liver. For example, during regeneration after partial hepatectomy, there are elevated levels of FN and α5 and β1 integrin subunits and decreased levels of gap junctions in the plasma membrane of hepatocytes prior to and during regeneration.[58] Our work here supports that role, as a blockade of α5β1 hinders viability of hepatocytes. It is also interesting to speculate if this increase in cell–matrix adhesions coincidental to a decrease in cell–cell adhesions during regeneration explains the ability of the cRGD peptide to inhibit formation of tissue-like aggregates as we find that bile canaliculi fail to form in the absence of FN fibrils and α5β1 signaling (Fig. 6 and Supplementary Figs. S4 and S5). *In vivo*, FN is observed at all hepatocyte plasma membrane domains: sinusoidal, lateral, and apical/canalicular.[55,58,59] However, during development and oncogenesis, the distribution of the nonintegrin FN receptor AGp110 correlates with the differentiation state of hepatocytes; AGp110 localization at the apical (canalicular) membrane indicates a differentiated state.[60–62] Hence, the loss of metabolic function in the absence of FN fibril formation and α5β1 signaling may be due to dedifferentiation resulting from the lack of cell polarity. It is also possible that more generalized signals from FN are crucial to the survival and differentiation of hepatocytes, without which the cells dedifferentiate and are rendered incapable of maintaining cell–cell contacts and other functions.

Growth factors also play a crucial role in liver development and regeneration, notably, HGF (ligand for proto-oncogenic mesenchymal epithelial transition factor [c-MET]), and EGFR ligands, including TGF-α and EGF. The relationship between these growth factors is complex, with a high level of redundancy amongst EGFR ligands as well as synergy with other growth factor receptors.[63,64] For instance, mouse knockouts of TGF-α or EGF show no impairment of liver development and loss of TGF-α does not impair liver regeneration. However, loss of EGFR—the signaling nexus for multiple extracellular ligands—causes impairment of liver regeneration in some models,[65,66] though not others,[67,68] wherein conflicting results may be attributed to differences in species, strain, and the specificity of silencing EGFR in hepatocytes. Although the effects of EGFR ablation on regeneration are equivocal, loss of c-MET, the receptor for HGF, dramatically impairs both embryonic development and liver regeneration.[65,69] In cultures where EGFR autocrine stimulation was blocked by mAb225, we observed a very significant loss of viability compared with control cultures (Fig. 4A, B); thus, *in vitro*, it appears that c-MET stimulation by autocrine HGF (Fig. 4 and Supplementary Fig. S3) does not completely compensate for the loss of EGFR signaling.

Determining the role of individual autocrine growth factors is further complicated by the potential for synergy or cooperativity with integrins. In a variety of cell types, it has been shown that integrin ligand binding and clustering result in increased growth factor receptor phosphorylation and enhanced signaling in shared downstream pathways.[70–73] In light of these facts, it is not surprising that hepatocytes in micromolded PEG–fibrinogen cultures require both integrin as well as growth factor signaling for proper survival and morphology as well as maintenance of differentiated metabolic functions. This system may be useful in further dissecting the intersecting and overlapping pathways between integrin and growth factor receptors that constitute the autocrine signaling network in the liver.

Conclusion

Autocrine matrix and growth factor regulation of primary rat hepatocyte survival and function in the absence of exogenous growth factors and adhesive ligands was studied using micropatterned PEG–fibrinogen hydrogels to provide an appropriate environment for 3D culture. Retention of autocrine-generated FN, TGF-α, EGF, and HGF by hepatocytes was observed in these 3D cultures, which assumed a tissue-like appearance and developed attachment to the walls of the microwells. Hepatocytes cultured in microwells adopted complex polarity including a functional bile canalicular network and maintained their viability and production of urea and albumin. Inhibition of α5β1 integrin binding to FN and inhibition of EGFR signaling in this culture format resulted in decreased hepatocyte survival and metabolic function and a decrease in soluble TGF-α, HGF, and EGF sequestration in the 3D tissue structure. Further, inhibition of α5β1 integrin showed a deficiency in fibrillar FN assembly, a disruption in the formation of tissue-like aggregates, and a failure of cells to polarize. Thus, this report indicates that autocrine matrix and growth factors are necessary and sufficient for maintenance of hepatocyte differentiation and survival *in vitro*.

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Disclosure Statement

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Hepatocyte induced re-expression of E-cadherin in breast and prostate cancer cells increases chemoresistance

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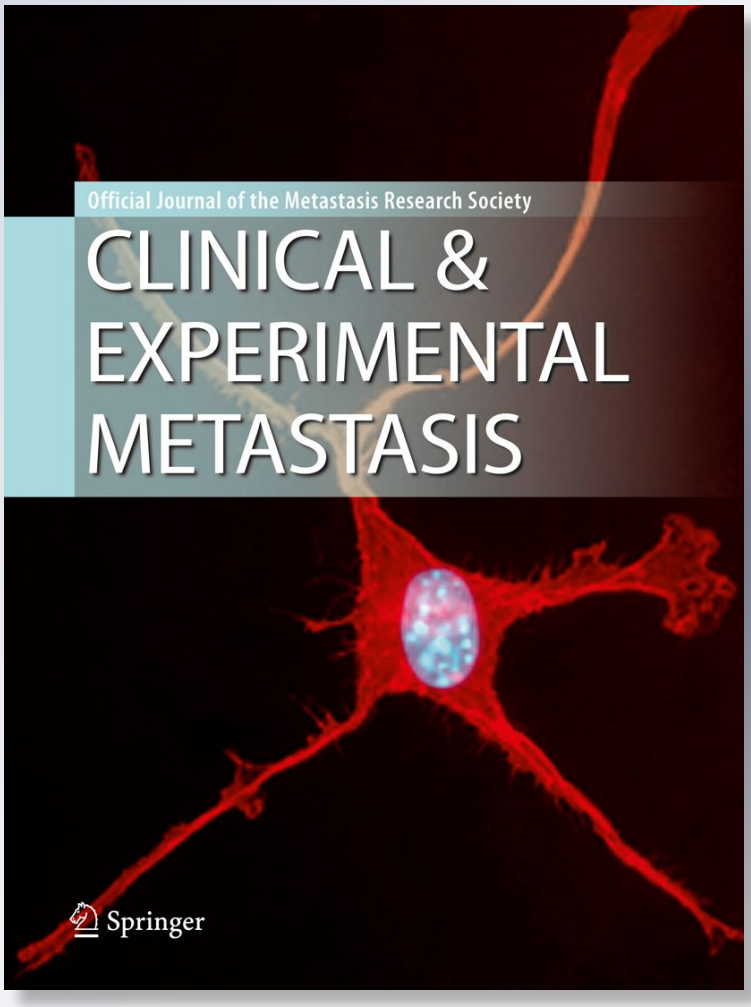

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RESEARCH PAPER

Hepatocyte induced re-expression of E-cadherin in breast and prostate cancer cells increases chemoresistance

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Abstract Post-extravasation survival is a key rate-limiting step of metastasis; however, not much is known about the factors that enable survival of the metastatic cancer cell at the secondary site. Furthermore, metastatic nodules are often refractory to current therapies, necessitating the elucidation of molecular changes that affect the chemosensitivity of metastases. Drug resistance exhibited by tumor spheroids has been shown to be mediated by cell adhesion and can be abrogated by addition of E-cadherin blocking antibody. We have previously shown that hepatocyte coculture induces the re-expression of E-cadherin in breast and prostate cancer cells. In this study, we show that this E-cadherin re-expression confers a survival advantage, particularly in the liver microenvironment. E-cadherin re-expression in MDA-MB-231 breast cancer cells resulted in increased attachment to hepatocytes. This heterotypic adhesion between cancer cells and secondary organ parenchymal cells activated ERK MAP kinase, suggesting a functional pro-survival role for E-cadherin during metastatic colonization of the liver. In addition, breast cancer cells that re-expressed E-cadherin in hepatocyte coculture were more chemoresistant compared to 231-shEcad cells unable to re-express E-cadherin. Similar results were obtained in DU-145 prostate cancer cells induced to re-express E-cadherin in hepatocyte coculture or following chemical induction by the GnRH agonist buserelin or the EGFR inhibitor PD153035. These results suggest that E-cadherin re-expression and other molecular changes imparted by a partial mesenchymal to epithelial reverting transition at the secondary site increase post-extravasation survival of the metastatic cancer cell and may help to elucidate why chemotherapy commonly fails to treat metastatic breast cancer.

Keywords Epithelial-to-mesenchymal transition · Mesenchymal-to-epithelial reverting transition · Chemoresistance · Cell death

Abbreviations

EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
GnRH agonist	Gonadotropin-releasing hormone agonist
MErT	Mesenchymal to epithelial reverting transition
PI-3k	Phospho-Inositide-3OH kinase
shRNA	Short hairpin RNA

Introduction

Approximately one-third of breast cancer patients will present with distant, non-nodal metastases, and as high as 60–70% of those patients will develop metastases in the liver [1, 2]. Breast cancer that metastasizes to the liver carries a very poor prognosis, with the median survival around 24 months [3]. Only 5% of patients with liver metastases present with a singular nodule; thus, surgical

Yvonne Chao and Qian Wu contributed equivalently to this work.

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resection is not an option for most. Current treatment for liver metastases relies on a multi-modal approach of systemic chemotherapy, endocrine- or HER2-targeted therapy if dictated by ER/PR/HER2 status, and palliative therapy such as radiation [4]. Poor response to chemotherapy is a major reason for the high mortality for breast cancer patients with liver metastases, and for all metastatic cancer patients in general. Elucidating the mechanisms behind chemoresistance in metastasis is therefore valuable for developing more effective therapies.

Just as not much is known about why metastases are refractory to chemotherapy, little is known about the molecular mechanisms controlling metastatic colonization of the liver. The liver is a major organ site for cancer metastases, so much so that liver metastases are more common than primary hepatic tumors [5]. A few of the cancers that exhibit organotropism to the liver include breast, prostate, and colorectal carcinomas [6]. Lumen occlusion or mechanical arrest in the first capillary bed encountered is insufficient for liver colonization [7, 8]. Selective cellular adhesion accounts for some of the organotropism exhibited by cancers, as cancer cell line variants that exhibit increased liver metastasis potential show increased adhesion to embryonic mouse liver cells [9]. Similarly, loss of claudins is associated with EMT whereas the upregulation of other tight junction components occurs in liver metastases. In vivo selection for a liver-aggressive variant of 4T1 breast cancer cells reveals that claudin-2 is upregulated in liver metastases and improves adhesion of the liver-aggressive cells to fibronectin and collagen IV, key components of the liver extracellular matrix (ECM) [10]. Selectins are a family of cell adhesion molecules that are differentially expressed on the vascular endothelial cells of various organs; colon cancer cells express different selectin ligands to adhere to particular organs [11, 12]. Expression of the epithelial-marker and cell adhesion molecule E-cadherin on breast cancer cells may be another mechanism to facilitate adhesion to hepatocytes, E-cadherin expressing parenchymal cells that account for 70–80% of the liver. Importantly, of the 4T1-derived cell lines with varying metastatic ability, only the 4T1 cells that express E-cadherin are able to form liver, lung, bone, and brain metastases while the E-cadherin-negative cell lines form only primary tumors [13, 14].

Besides mediating physical adhesion to organ parenchymal cells to facilitate colonization, expression of E-cadherin is also associated with cell survival. Expression of E-cadherin on hepatocyte spheroids in culture protects against detachment-induced cell death, or anoikis, in a caspase-independent manner [15]. This is consistent with a report that endocytosis of E-cadherin induced by EGFR activation leads to anoikis of enterocytes [16]. The assembly of adherens junctions coordinated by E-cadherin ligation quickly leads to sustained activation of MAPK and Akt, in a mode of signaling for these pathways that leads to cell survival rather than proliferation [17, 18]. The related cadherin family member VE-cadherin likewise controls endothelial cell survival through signaling through Akt and Bcl-2 [19]. Thus, breast cancer cells may activate survival signaling through heterotypic ligation with hepatocytes.

We have shown previously that the liver microenvironment induces the re-expression of E-cadherin in breast and prostate cancer cells [20, 21]. Thus, the aim of this study was to determine whether there is a functional significance to E-cadherin re-expression. We show that E-cadherin promotes attachment to the secondary organ parenchymal cells through heterotypic ligation, with this resulting in the sustained activation of ERK MAP kinase. Furthermore, E-cadherin re-expression also confers a functional survival advantage by increasing the resistance of breast and prostate cancer cells to chemotherapy-induced cell death in the liver microenvironment.

Results

E-cadherin expression affects survival through heterotypic adhesion of breast cancer cells to hepatocytes

E-cadherin-negative MDA-MB-231 breast cancer and DU-145 prostate cancer cells re-express E-cadherin and revert to an epithelial morphology when cocultured with rat hepatocytes, a cell culture model for the liver microenvironment [20, 21]. These results were also observed upon coculture with lung parenchymal cells [22]. As mediating intercellular adhesion is a major function of E-cadherin, we hypothesized that post-extravasation survival of cancer cells at the secondary site is facilitated by heterotypic adhesion between cancer cells and organ parenchymal cells. To probe this role we used previously characterized E-cadherin knock-in and knock-down lines: E-cadherin-negative MDA-MB-231 cells (231), MDA-MB-231 cells that exogenously express E-cadherin (231-Ecad), MDA-MB-231 cells stably expressing E-cadherin shRNA (231-shEcad), E-cadherin-positive MCF7 cells, and MCF7 cells stably expressing E-cadherin shRNA (MCF7-shEcad). All cell lines were RFP-labeled to facilitate detection of cancer cells in hepatocyte coculture. When cocultured with human hepatocytes for 6 days, 231 cells reverted to an epithelial morphology and re-expressed E-cadherin (Fig. 1) (similar reversion was noted with rat hepatocytes, data not shown). In contrast, an analogous phenotypic change was not observed in cocultured 231-shEcad cells. The phenotypic effect of this change was mirrored in the cell

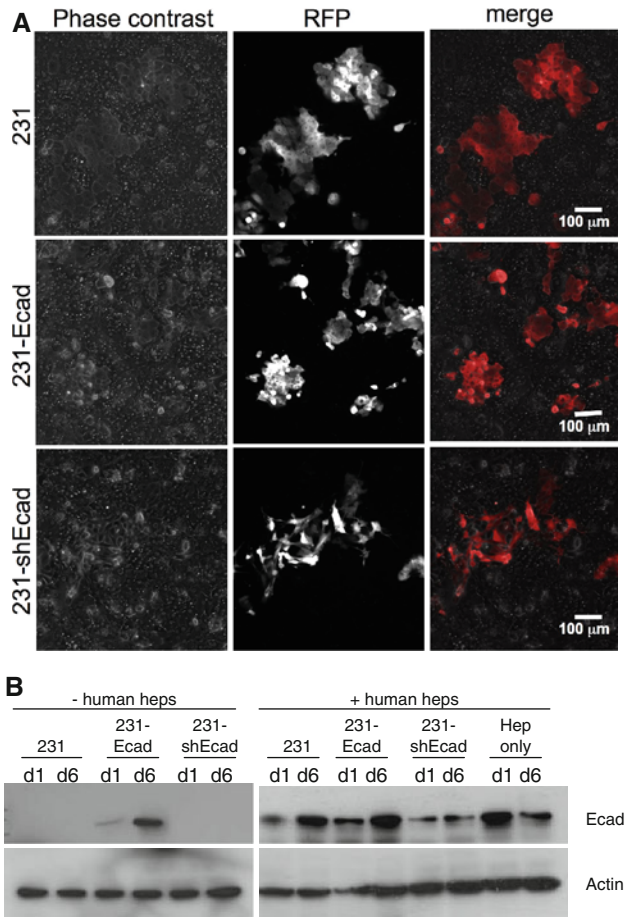

Fig. 1 Breast cancer cells cultured with hepatocytes revert to epithelial cluster morphology and re-express E-cadherin. **a** Phase contrast and fluorescent images of breast cancer cells cocultured with hepatocytes for 6 days. **b** Immunoblot of E-cadherin expression in breast cancer cells cultured with and without human hepatocytes. Shown are representative of three experiments

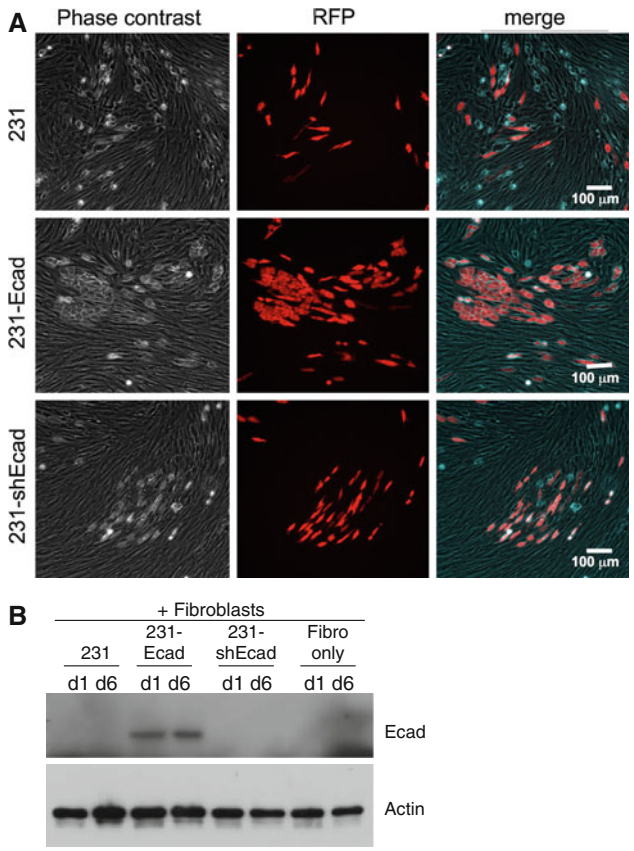

Fig. 2 Breast cancer cells cultured with fibroblasts fail to re-express E-cadherin. **a** Phase contrast and fluorescent images of breast cancer cells cocultured with fibroblasts for 6 days. **b** Inmunoblot of E-cadherin expression in breast cancer cells cultured with human fibroblasts. Shown are representative of two experiments

distribution pattern in which the E-cadherin-expressing cells (231-Ecad, and 231 after coculture) clustered, suggesting cell–cell contacts, whereas the E-cadherin-negative cells (231-shEcad) remained as single cells interspersed among the hepatocytes. The three cell lines were also cocultured with primary human fibroblasts. Following 6 days of fibroblast coculture, 231 cells remained mesenchymal in phenotype and singularly interspersed (Fig. 2). These cells remained E-cadherin negative, demonstrating that the re-expression is dependent on the hepatocytes (Fig. 2).

To test whether attachment to hepatocytes was mediated by E-cadherin expression, hepatocytes (which express E-cadherin) or fibroblasts (which lack E-cadherin) were plated on collagen-coated plates at 30% confluency and cancer cells were seeded onto the monolayer the following day. Four hours later, the number of RFP-positive cells in the monolayer was counted as a measure of attachment. On the hepatocyte monolayer, E-cadherin-positive cells 231-Ecad and MCF7 exhibited significantly increased attachment compared to E-cadherin-negative cells ($P = 0.05$). (Fig. 3a). However, when cultured on the fibroblast monolayer to account for nonspecific adhesion and adhesion to exposed collagen matrix there was no statistically significant difference in attachment between the various cell lines. However, it was possible that the differences in attachment were not entirely E-cadherin dependent, as the plating of hepatocytes and fibroblasts at 30% confluency left portions of the collagen-coated plastic exposed. As a result, the cell lines were plated on densities ranging from 25 to 100% confluency. Thus, at higher hepatocyte densities attachment would only be generated by cancer cell adhesion to the hepatocyte monolayer. As expected, the ability of E-cadherin-positive 231-Ecad and MCF7 cells to attach was not diminished by hepatocyte density while attachment of E-cadherin-negative 231 and MCF7-shEcad cells decreased with increasing hepatocyte density ($P < 0.05$ between 25 and 100%) (Fig. 3b, d). In contrast, attachment of all cell lines decreased with increasing density of fibroblasts, further confirming that cancer cell–hepatocyte attachment is mediated by E-cadherin (Fig. 3c, e). While lack of E-cadherin expression

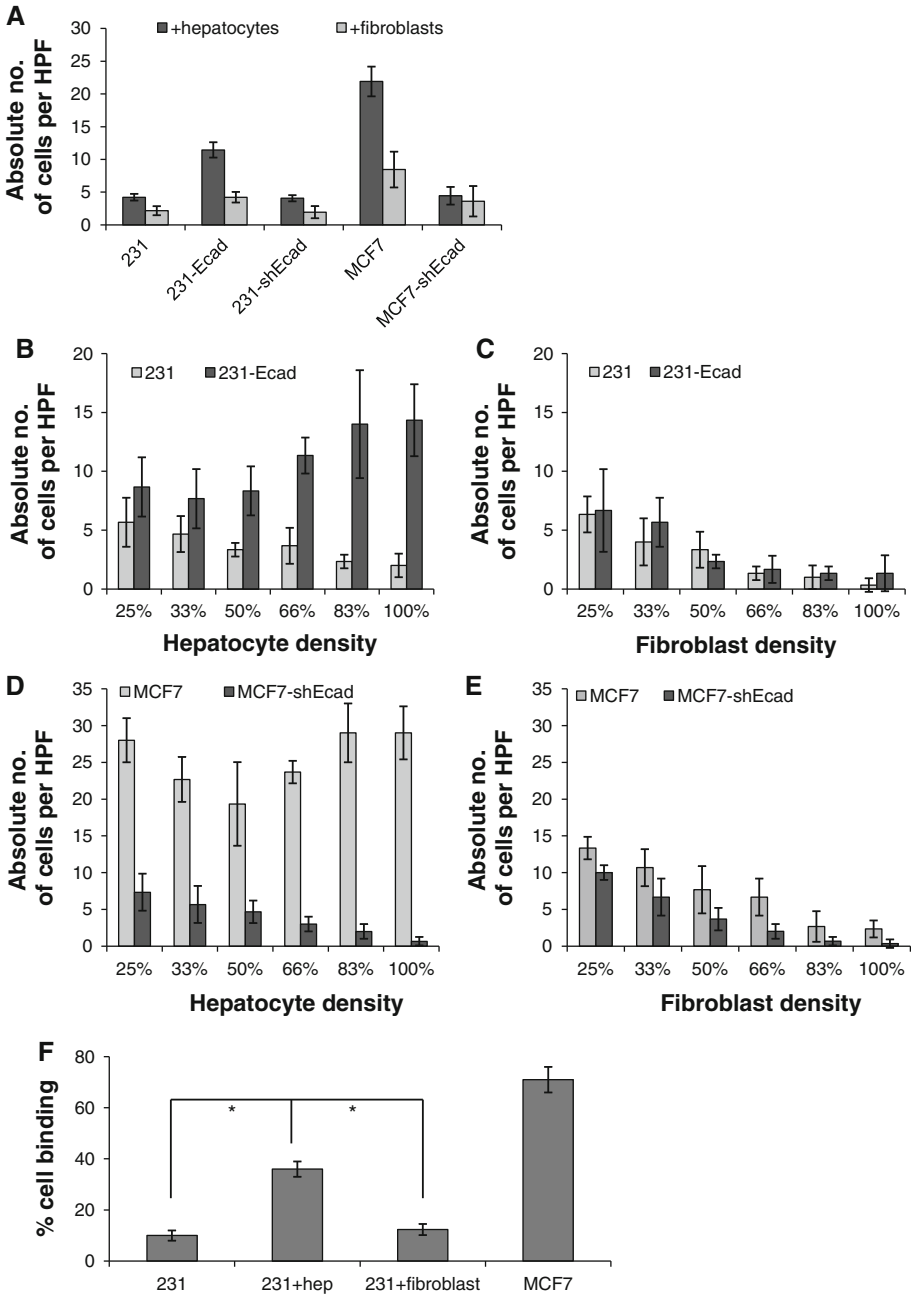

Fig. 3 E-cadherin expression increases attachment to hepatocytes. **a** Attachment of E-cadherin-negative and -positive breast cancer cells to hepatocytes or fibroblasts plated at 30% confluency. **b**, **c** Attachment of 231 and 231-Ecad breast cancer cells to hepatocytes or fibroblasts plated at 25–100% confluency. **d**, **e** Attachment of MCF7 and MCF7-shEcad breast cancer cells to hepatocytes or fibroblasts plated at 25–100% confluency. **f** Binding of breast cancer cells to hepatocytes or fibroblasts by centrifugal assay for fluorescence based cell adhesion. Shown are mean ± sem. ($n = 3$, in triplicate) ($P \leq 0.05$)

initially impeded the ability of 231 cells to attach to hepatocytes, re-expression of E-cadherin in 231 cells following 6 days of hepatocyte coculture increased attachment, as measured by a centrifugal assay for fluorescence-based cell adhesion (CAFCA). This adhesion was not observed when 231 cells were cocultured with fibroblasts (Fig. 3f). Thus, the re-expressed E-cadherin was capable of establishing heterotypic cell–cell adhesions. Control experiments using MCF7 cells revealed that the heterotypic attachment between breast cancer cells and hepatocytes is E-cadherin dependent, as addition of the E-cadherin blocking antibody SHE78, calcium chelator EDTA, and E-cadherin siRNA all limited cell binding to hepatocytes as assessed by CAFCA (Supplemental Figure 1).

E-cadherin homotypic ligation activates survival signaling pathways [17, 18], so next we queried whether heterotypic ligation between breast cancer cells and hepatocytes resulted in similar activation. To isolate signaling only occurring in the breast cancer cells (apart from the cognate hepatocyte partner), hepatocyte membranes were isolated and adsorbed onto culture plates and labeled with DiI (Supplemental Figure 2a). Activation of the Erk MAP kinase pathway was probed after MCF7 cells and MDA-MB-231 cells cultured with and without hepatocytes for

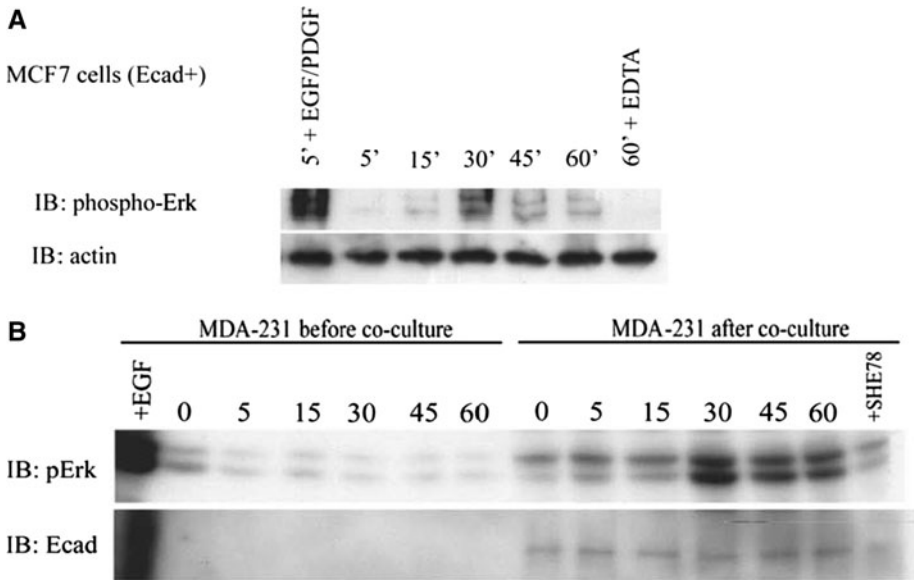

Fig. 4 Heterotypic ligation between breast and prostate cancer cells activates Erk signaling (**a**) E-cadherin-positive MCF7 cells plated onto hepatocyte membranes; addition of EDTA prevents Erk activation (**b**) 231 cells with and without E-cadherin re-expression plated onto hepatocyte membranes; addition of E-cadherin blocking antibody SHE78 blocks Erk signaling in 231 cells that re-express E-cadherin. Shown are representative of at least three experiments

6 days were plated onto hepatocyte membranes. Maximal phospho-Erk expression was detected 30 min after plating E-cadherin-positive MCF7 cells onto hepatocyte membranes (Fig. 4a). Erk activation was not observed in E-cadherin-negative 231 cells cultured in the absence of hepatocytes, but was observed 30 min after addition of E-cadherin re-expressing 231 cells (Fig. 4b). Activation of Erk signaling was dependent on E-cadherin ligation as addition of E-cadherin blocking antibody SHE78 blocked the increase in pErk (Fig. 4b). Heterotypic ligation of MCF7 cells and hepatocytes also activated Akt (Supplemental Figure 2), suggesting that survival pathways in addition to Erk MAP kinase may be involved.

E-cadherin expression increases chemoresistance of breast and prostate cancer cells

Multiple studies have suggested that E-cadherin ligandation protects against cell death and increases drug resistance of tumors [23–25]. To evaluate chemoresistance, we tested the cytotoxic effect of various chemotherapeutic agents commonly used to treat breast cancer on our cell lines. Likely because 231 cells were derived from a patient who was treated with many of these agents, these cells were only slightly sensitive at best to many of the drugs tested: 5-fluorouracil, cyclophosphamide, doxorubicin, and taxol (Supplemental Figure 3). We therefore selected the protein kinase inhibitor and apoptosis-inducer staurosporine and DNA topoisomerase inhibitor camptothecin to induce cancer cell death in the following studies; these agents are representatives of two categories of chemotherapy drugs. The TC50 was 54.18 nM for staurosporine and 6.35 μM for camptothecin (Supplemental Figure 4a and 4b); therefore, a range of 0.01–1,000 nM staurosporine and a range of 0.001–100 μM camptothecin was used for cell survival analysis. Treatment of breast cancer cells with staurosporine and camptothecin showed that 231-Ecad cells were less sensitive to cell death induced by these agents compared to E-cadherin negative 231 and 231-shEcad cells (Fig. 5a, c). Addition of E-cadherin antibody abrogated the effect on 231-Ecad cells (Supplemental Figure 5). The TC50s for staurosporine and camptothecin treatment of 231-Ecad was higher than for 231 and 231-shEcad cells, further demonstrating the chemoprotection effects from E-cadherin expression in breast cancer cells (Supplemental Figure 4). Similar results were observed in breast cancer cells treated with other chemotherapeutic drugs taxol and doxorubicin (Supplemental Figure 3c and 3d).

As noncycling cells are more difficult to kill than cycling cells, the findings of limited chemoprotection may simply reside from differences in mitogenesis between the lines. This was not the case, as all lines proliferated and expanded indistinguishably (Supplemental Figure 6).

To determine whether this chemoprotection was unique to breast cancer cells, we corroborated these results in prostate cancer cells chemically induced to express E-cadherin. We have previously shown that prostate cancer cells also re-express E-cadherin upon coculture with parenchymal cells of target organs [20, 22], or even just repression of EGFR signaling by EGFR kinase inhibition by a direct agent (PD153035) or indirectly by a gonadotropin-releasing hormone (GnRH) agonist [20, 26]. DU-145 prostate cancer cells were treated with 1 μg/ml of the GnRH agonist buserelin or 500 nM EGFR kinase inhibitor PD153035 for 48 h. Treatment with these agents resulted in re-expression of E-cadherin and an epithelial cluster morphology (Fig. 6a, b). Following E-cadherin re-expression induced by these agents, DU-145 cells were more resistant to cell death

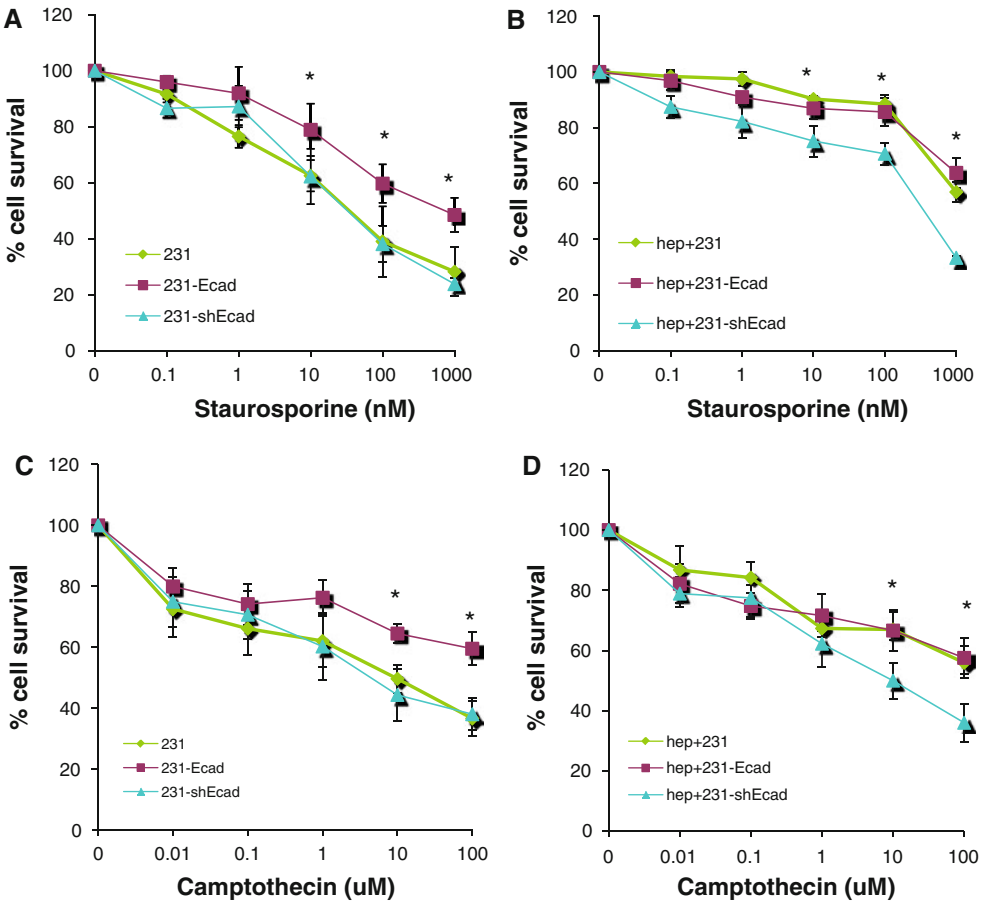

Fig. 5 Exogenous and microenvironment-induced expression of E-cadherin in breast cancer cells increases the chemoresistance to staurosporine (**a**, **b**) and camptothecin (**c**, **d**). Shown are mean ± sem. ($n = 3$, in triplicate) ($P < 0.05$)

induced by staurosporine and camptothecin (Fig. 6c, d). Addition of E-cadherin blocking antibody abrogated the effect on DU-145 cells (Supplemental Figure 7), indicating the chemoprotection was a result of E-cadherin re-expression. At least part of the limited degree of noted protection can be explained by the fact that not all of the prostate cancer cells re-express E-cadherin under the treatment (Fig. 6b).

To understand the mechanism behind the chemoprotection exhibited by E-cadherin-positive cells, caspase 3 activity was assessed in 231, 231-Ecad and 231-shEcad cells following staurosporine or camptothecin treatment (Supplemental Figure 8). Mild reduction of caspase3 activity was observed in 231-Ecad cells compared to 231 and 231-shEcad cells after drug treatment, suggesting E-cadherin re-expression diminishes activation of apoptotic signals in breast cancer cells.

E-cadherin re-expression in the liver microenvironment increases the chemoresistance of breast and prostate cancer cells

The above provides a proof of concept of chemoprotection by E-cadherin, one that is consistent with literature reports [25, 27]. However, the extent of chemoprotection is modest, but this could simply be due to the artificial and limited extent of epithelial reversion based solely on exogenous induction of E-cadherin expression. Thus, we tested whether similar chemoprotection could be effected in the liver microenvironment. On day 6 of hepatocyte coculture, breast and prostate cancer cells were treated with staurosporine and camptothecin and the number of surviving RFP-positive cells were counted after a further 24 (staurosporine) or 48 h (camptothecin). E-cadherin re-expression in hepatocyte coculture increased the chemoresistance of 231 cells to 231-Ecad levels, while 231-shEcad cells unable to re-express E-cadherin remained the most sensitive (Fig. 5b, d). Interestingly, overall the breast cancer cells were less sensitive to staurosporine treatment in hepatocyte coculture as the IC50 was tenfold higher in coculture, which may be explained by molecular changes besides E-cadherin re-expression that allow for a more complete reversion to the epithelial phenotype not observed when only E-cadherin is exogenously expressed.

DU-145 prostate cancer cells were also induced to heterogeneously re-express E-cadherin in the liver microenvironment (Fig. 7a, Supplemental Figure 9a). A notable increase of E-cadherin expression localized to the

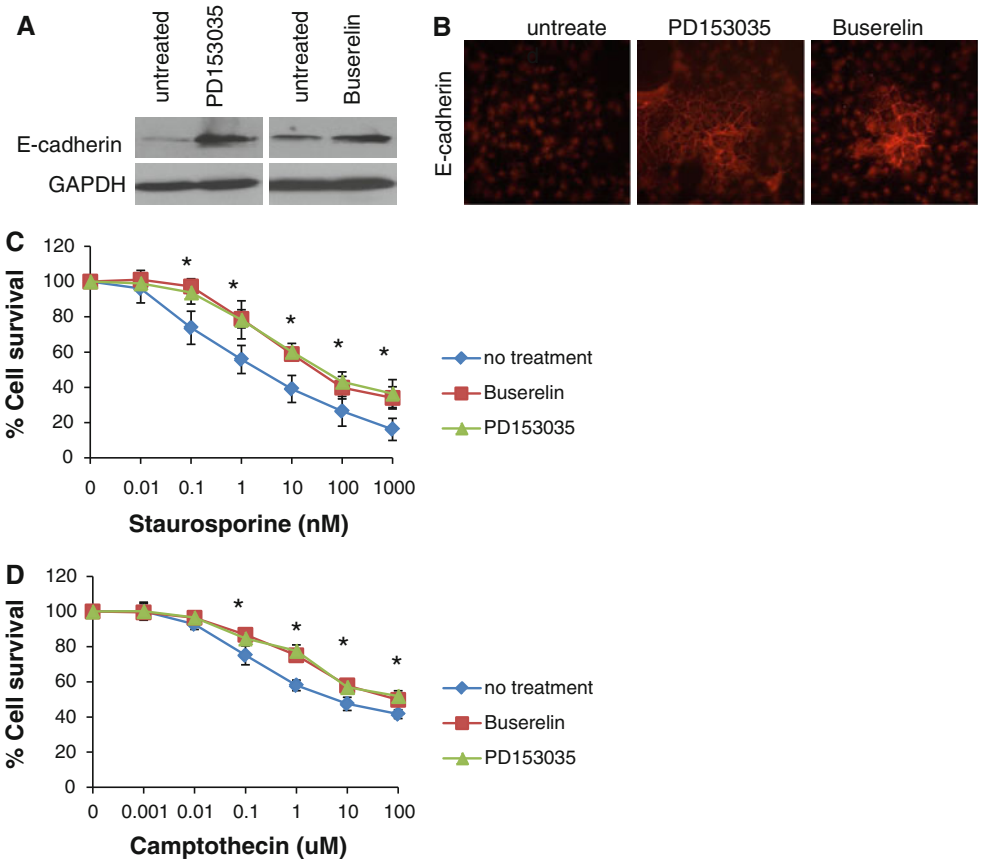

Fig. 6 E-cadherin re-expression in prostate cancer cells increases chemoresistance. **a** Immunoblot for E-cadherin following treatment with buserelin or PD153035. **b** Immunofluorescence for E-cadherin following treatment with buserelin or PD153035. **c** DU-145 cells treated with camptothecin (**c**) and staurosporine (**d**) with or without re-expression of E-cadherin by buserelin and PD153035. In **a** and **b**, shown are representative of three experiments. In **c** and **d**, shown are mean ± sem. ($n = 3$, in triplicate) ($P < 0.05$)

membrane of DU-145 cells was observed after coculture with human hepatocytes (Fig. 7b, c). This heterogeneous E-cadherin re-expression also exhibited increased resistance to cell death (Fig. 7d, e). The increased chemoresistance was abrogated when DU-145 cells were transiently transfected with E-cadherin siRNA prior to coculture (Fig. 7d, e and Supplemental Figure 9d). Because primary isolation of hepatocytes often includes fibroblasts and other non-parenchymal cells, to show that this protective effect was mediated by E-cadherin re-expression induced by the hepatocytes, the chemosenstivity of prostate cancer cells following coculture with fibroblasts was also tested. No E-cadherin re-expression was observed in DU-145 and fibroblast coculture (Supplemental Figure 9b). Following staurosporine and camptothecin treatment, the level of chemosensitivty of DU-145 cells cocultured with fibroblasts was similar to DU-145 cells cultured in the absence of hepatocytes (Fig. 7d, e).

There remains the question of whether the chemoprotection noted in the presence of the liver microenvironment is due to metabolism of the agents by the hepatocytes. It should be noted that hepatocytes in two-dimensional culture, as performed here in the cocultures, lose metabolic capacity over time with little remaining after 6 days [28–30] and therefore would not likely be active metabolizers. Still, this needed to be addressed experimentally. The prostate carcinoma cells were cocultured with hepatocytes isolated in a transwell system, which does not allow for epithelial reversion (Supplemental Figure 9c) though hepatocyte metabolism of agent would still occur; in this situation, there was no evidence of chemoprotection (Fig. 7d, e). Similar protection was obtained in DU-145 cells treated with cisplatin, a chemotherapy drug, after E-cadherin re-expression induced by hepatocyte coculture (Supplementary Figure 10).

Discussion

Alterations in adhesion have been shown to be necessary for many steps of metastasis, from down-regulation of E-cadherin in EMT during invasion to expression of selectin ligands or gap junction molecules for adherence to endothelial cells during extravasation [7, 9, 12, 31]. We have shown previously that metastatic tumors from breast and prostate cancer patients express increased levels of E-cadherin compared to the primary tumor, which is accompanied by a partial mesenchymal to epithelial reverting transition [20, 21, 32]. Furthermore, E-cadherin re-expression is also observed when cultured in a liver

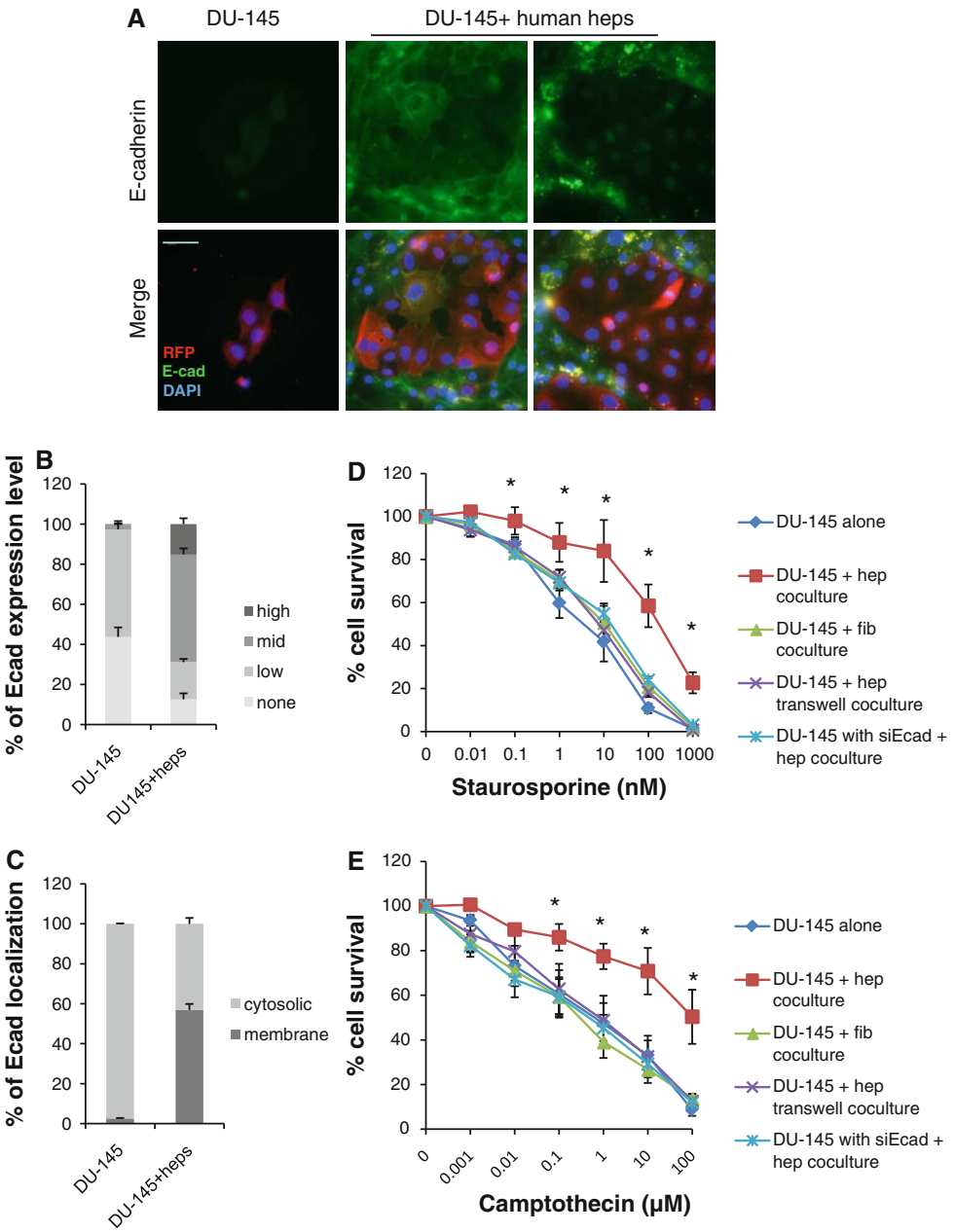

Fig. 7 Prostate cancer cells that re-express E-cadherin in hepatocyte coculture are more chemoresistant to staurosporine and camptothecin. **a** E-cadherin re-expression in DU-145 cells after hepatocyte coculture. DU-145 cells are labeled by RFP. E-cadherin is shown in the upper panels using specific immunostaining. *Left column*, DU-145 only; *Middle column*, an example of E-cadherin re-expression in DU-145 cells with coculture; *Right column*, an example of negative E-cadherin expression in DU-145 cells with coculture. **b** Quantification of percentage of DU-145 cells with none, low, mid and high levels of Ecadherin. **c** Quantification of E-cadherin localization in DU-145 cells with and without hepatocytes coculture. **d**, **e** DU-145 cells are more chemoresistant to **d** staurosporine and **e** camptothecin.This effect is abrogated in fibroblast coculture or when cells are transiently transfected with E-cadherin siRNA prior to coculture, or transwell coculture with hepato. Shown are mean ± sem. ($n = 3$, in triplicate) ($P \leq 0.05$)

microenvironment in vitro and in lung metastases in an in vivo animal model [21]. Our findings herein show that the functional significance of E-cadherin expression in metastases may be to increase attachment and integration within organ parenchyma, and to subsequently increase post-extravasation survival through E-cadherin-mediated survival signaling. Besides physical intercellular adhesion, E-cadherin engagement also activates internal signaling pathways that promote survival through suppression of anoikis and canonical Erk and Akt pathways [17, 18]. E-cadherin binding of epithelial cells has also been shown to promote survival in a PI-3K-dependent fashion [33]. The finding that Erk is phosphorylated upon binding to hepatocytes by re-expressed E-cadherin on MDA-MB-231 cells implies that relevant functional signaling occurs as a result of heterotypic ligation between cancer cells and organ parenchymal cells.

A critical result of this reversion to a more epithelial phenotype is the resistance to induced cell death. Previous studies have shown the protective role of E-cadherin in the face of chemotherapy and our studies corroborate these results [23]. Of particular interest is the finding that breast and prostate carcinoma cells in hepatocyte coculture were more resistant to cell death-induced by staurosporine or camptothecin compared to cells cultured in the absence of hepatocytes. This is not due to hepatocyte metabolism of

agents independent of the phenotypic reversion as shRNA to E-cadherin blunts this coculture protection, and coculture without physical juxtaposition, which does not alter the carcinoma cell phenotype, did not confer chemoprotection. While it remains to be experimentally dissected, we propose that the normal parenchymal cells induce a more complete phenotypic shift. We have shown evidence that a partial mesenchymal to epithelial reversion occurs in human breast and prostate cancer metastases, suggesting that the liver microenvironment can induce other molecular changes besides E-cadherin expression during partial MErT [21, 32]. One such change can be re-expression of the gap junction protein connexins, which are frequently downregulated in EMT and have been shown to be upregulated in lymph node metastases; hepatocyte coculture induces re-expression of connexin43 in breast cancer cells (data not shown). Brain metastases of breast cancer patients exhibit increased expression of E-cadherin, Cx43 and Cx 26 [32]. A recent study showed that astrocyte-cancer cell interactions mediated by gap junction expression protects cancer cells from chemotherapy-induced cell death [34, 35]. Thus adhesion, facilitated by gap junctions in this case, promotes the survival of cancer cells during metastatic colonization.

Some of the functional mechanisms behind the increased chemoresistance in E-cadherin re-expressing cells in our model have been revealed. Pro-survival pathways such as Erk MAP kinase and Akt are noted as activated upon E-cadherin re-expression. Akt signaling also contributes to chemoresistance [36]. Furthermore, we showed decreased activity of apoptosis effector caspase 3 in E-cadherin-positive cells, providing a second possible mechanism for the chemoprotection. Other studies suggest that anti-apoptotic proteins such as Bcl-2 or cell cycle inhibitors cyclin-dependent kinase inhibitor p27 may also be involved [25, 37]. Another potential explanation for the increased chemoresistance is contact mediated growth inhibition governed by E-cadherin [38]; however, growth inhibition of MDA-MB-231 cells upon re-expression of E-cadherin was not observed in either 231-Ecad cell lines or hepatocyte coculture (data not shown). Deeper molecular dissection of the operative pathways underlying this chemoprotection lies beyond the scope of the present manuscript, but remains a key area for further investigation.

Also remaining is the question of whether E-cadherin expression is required for the initial establishment of metastases. E-cadherin re-expression could explain the propensity for breast and prostate cancer cells to metastasize to lung and liver, both lined with epithelial cells expressing this cell recognition molecule. In support of a proposed cell–cell recognition moiety is that fact that aberrant expression of osteoblast cadherin, also known as OB-cadherin and cadherin-11, on breast and prostate cancer cells, increases metastases to the bone by increasing migration and intercalation with osteoblasts [39, 40]. It is also possible that the chemoprotection conferred by E-cadherin re-expression and ligandation also promotes the survival of disseminated carcinoma cells in the face of a challenging ectopic environment or any intrinsic inflammatory response upon metastatic seeding.

This transitional step opens the role of phenotypic plasticity in tumor progression and the metastatic cascade. It is well-established that E-cadherin functions as a 'tumor suppressor' and its forced expression limits metastatic dissemination. Thus, the ability of E-cadherin to support metastasis has been brought into question [41]. Of interest, the phenotypic reversion to a more-epithelial phenotype is driven by the receptive microenvironment. Coculture of cancer cells with normal fibroblasts failed to produce the epithelial reversion and concomitant re-expression of E-cadherin, further suggesting that the phenotypic changes of the cancer cell reflect the microenvironment. An inverse correlation of E-cadherin with size of metastases suggests that this phenotypic reversion is not stable, and would only be advantageous for small micrometastases [32]. There are several therapeutic implications raised by this study, even with a number of open questions as noted above. Expressing E-cadherin or attempting to revert carcinoma phenotype towards a more epithelial state, while limiting escape from the primary tumor site, may perversely improve metastatic competency of the multitude of shed cells. On the other hand, downregulating E-cadherin would likely make the carcinomas more invasive and aggressive. As metastases constitute the major part of carcinoma mortality, new approaches should target the micrometastases to kill them prior to frank metastatic disease. Thus, the survival signals activated upon heterotypic E-cadherin ligation or the as yet unknown microenvironmental cues that initially induce expression of E-cadherin in the secondary organ may thus be the more effective therapeutic targets.

Materials and methods

Cell lines and cell culture

231-RFP, 231-Ecad-RFP, and 231-shEcad-RFP breast cancer cells and DU-145 prostate cancer cells were cultured in RPMI as previously described [21]. Selected cells were isolated on more than one occasion with little difference between the selections; transient transfections also provided similar cell responses but were not used due to the cell–cell heterogeneity making cell quantitation difficult (data not shown). Human fibroblasts 10-1169F were cultured in DMEM.

Coculture

Primary rat and human hepatocytes were isolated and plated at 4×10^5 cells per well in 6-well plates coated with 10% rat tail collagen in dH_2O (BD Biosciences) at 30% confluency and allowed to attach overnight. The next day, 2×10^4 RFP-labeled cancer cells were seeded onto hepatocyte monolayers. Rat cocultures were maintained in Hepatocyte Growth Media (HGM) and human hepatocytes were maintained with Hepatocyte Maintenance Media (Lonza). For fibroblast cocultures, the fibroblast monolayer was initially plated at 1×10^5 cells per well in 6-well plates and seeded with 2×10^4 the following day. Media was replenished daily. For transwell coculture, inserts (Millipore) was coated with 10% rat tail collagen and plated with hepatocytes at 4×10^5 cells per insert. Cancer cells were seeded with 2×10^4 in the bottom chamber the following day. Cells were treated or collected for analysis after 5-day transwell coculture.

Chemical re-expression of E-cadherin

DU-145 cells were seeded in 96-well plates and treated with 1 μg/ml buserelin or 500 nM PD153035 for 48 h. Immunoblot and immunofluorescence to confirm E-cadherin expression was performed using E-cadherin antibody (Cell Signaling). E-cadherin blocking antibody was used at 5 μg/ml (Invitrogen).

Attachment assay

Primary hepatocytes were plated at densities ranging from 25 to 100% confluency on collagen-coated 6-well plates and allowed to attach overnight. The next day, 2E4 RFP-labeled cancer cells were seeded in each well. Four hours later, wells were washed once with PBS to remove any unattached cells and the number of RFP positive cells in each well was quantified.

Centrifugal assay for fluorescent cell adhesion (CAFCA)

Cancer cells were non-enzymatically dissociated and labeled with 5 M Calcein AM (Molecular Probes, Carlsbad, CA, USA). Labeled cancer cells were seeded at a density of 42,000 cells well in 96-well plates containing a densely confluent hepatocyte monolayer. The plates were centrifuged for <60 s at $50\times g$ to pellet the cancer cells onto the hepatic monolayer, then incubated at 37°C. At defined times, the plates were inverted and centrifuged at $600\times g$ for 5 min and then gently washed to remove unbound cells from the hepatocyte monolayer. Fluorescence was measured with a 494/517 bandpass filter set-up from the bottom of the plate by a TECAN Spectra-Fluor plate fluorometer. Absolute emission measurements were background subtracted.

Chemoprotection assay

For cell death assays in the absence of hepatocytes, breast and prostate cancer cells were seeded in 96-well plates and treated with 0–1,000 nM of staurosporine for 24 h or 0–100 μM of camptothecin for 48 h. Wells were then stained with 1 μM calcein AM for 30 min and fluorescence was quantified with Tecan Spectrafluor. In the presence of hepatocytes, following induction of cell death with staurosporine or camptothecin, the number of RFP-positive cells in each well was counted.

Hepatocyte membrane assay

Culture plates were coated with poly-L-lysine (Sigma) and hepatocyte membranes (2 mg protein/cm^2) were allowed to adsorb onto poly-L-lysine-coated 6-well plates for 10 min. Hepatocyte membranes were labeled with DiI (Molecular Probes) for visualization. MDA-MB-231 cells were sorted from hepatocyte cocultures and quiesced in serum-free media for 3 h, then seeded 2E4 cells onto the membrane coated plates and centrifuged at $50\times g$ for 1 min. RIPA lysates were taken at each time point and pErk (Santa Cruz Biotech) was detected by immunoblot.

TC50 analysis

Breast and prostate cancer cells were plated in 96-well plates and treated with 0–1 μM of staurosporine for 24 h or 0–1,000 μM of camptothecin for 48 h. Vybrant MTT cell proliferation assay was performed according to the manufacturer's protocol (Invitrogen).

Caspase 3 activity assay

Breast cancer cells were seeded in 8-well chamber slides and treated with 10 and 100 nM of staurosporine for 8 h or 10 and 100 μM of camptothecin for 16 h. Caspase 3 activity was measured by CaspaTag Caspase 3 in situ assay kit according to the manufacture's protocol (Chemicon).

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ORIGINAL PAPER

Partial Mesenchymal to Epithelial Reverting Transition in Breast and Prostate Cancer Metastases

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Abstract Epithelial to mesenchymal transition (EMT) is an oft-studied mechanism for the initiation of metastasis. We have recently shown that once cancer cells disseminate to a secondary organ, a mesenchymal to epithelial reverting transition (MErT) may occur, which we postulate is to enable metastatic colonization. Despite a wealth of in vitro and in vivo studies, evidence supportive of MErT in human specimens is rare and difficult to document because clinically detectable metastases are typically past the micrometastatic stage at which this transition is most likely evident. We obtained paired primary and metastatic tumors from breast and prostate cancer patients and evaluated expression of various epithelial and mesenchymal markers by immunohistochemistry. The metastases exhibited increased expression of membranous E-cadherin compared to primary tumors, consistent with EMT at the primary site and MErT at the metastatic site. However, the re-emergence of the epithelial phenotype was only partial or incomplete. Expression of epithelial markers connexins 26 and/or 43 was also increased on the majority of metastases, particularly those to the brain. Despite the upregulation of epithelial markers in metastases, expression of mesenchymal markers vimentin and FSP1 was mostly unchanged. We also examined prostate carcinoma metastases of varied sizes and found that while E-cadherin expression was increased compared to the primary lesion, the expression inversely correlated with size of the metastasis. This not only suggests that a second EMT may occur in the ectopic site for tumor growth or to seed further metastases, but also provides a basis for the failure to discern epithelial phenotypes in clinically examined macrometastases. In summary, we report increased expression of epithelial markers and persistence of mesenchymal markers consistent with a partial MErT that readily allows for a second EMT at the metastatic site. Our results suggest that cancer cells continue to display phenotypic plasticity beyond the EMT that initiates metastasis.

Keywords Mesenchymal-to-Epithelial transition
E-cadherin · Differentiation · Connexin

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Introduction

Recapitulation of the developmental process of epithelial to mesenchymal transition (EMT) has been proposed as a mechanism for enabling cancer cell invasion and dissemination. During cancer-associated EMT, loss of cell-cell adhesions via downregulation of E-cadherin allows for both physical detachment from the tumor mass and for external autocrine growth factor and internal signaling that activates cell migration [1]. EMT in cancer progression and metastasis has been widely studied through in vitro cell culture and in vivo animal models of cancer progression. In addition, EMT has been visualized at the invasive front of primary carcinomas as individual cells or a group of cells migrating into the surrounding tissue [2]. However, the true extent of EMT in human cancer specimens is still open to debate as is the role of EMT in metastatic seeding [1, 3, 4].

Despite the strong clinical association between decreased expression of adhesion molecules and invasion and poor prognosis, metastases can present a well-differentiated,

epithelial phenotype, bringing into question whether EMT is reversible. We and others have proposed that a reverse EMT, or mesenchymal to epithelial reverting transition (MErT), occurs to enable metastatic colonization [4–7]. Therefore, while induction of EMT through loss of E-cadherin may promote tumor invasion and dissemination, MErT through re-expression of epithelial genes and downregulation of mesenchymal genes may allow the metastatic cancer cell to complete the last steps of the metastatic process and to survive in the secondary organ. However, just as it has been difficult to capture EMT in vivo, there is also a dearth of histological evidence for MErT.

Opponents of cancer-associated EMT argue that there is a lack of convincing evidence in clinical samples that support the in vitro findings [3]. However, lack of evidence in clinical samples does not mean that an EMT or MErT has not occurred at some point in time, as pathological specimens are often end-stage observations. Unless clinically indicated, only a small percentage of metastases undergo surgical resection or biopsy, as systemic adjuvant endocrine, chemotherapy, or palliative radiation is more commonly used as therapy. Furthermore, specimens of metastases that are resected or that undergo biopsy originate from tumors of various stage and size (and ER/PR/HER2/neu status for breast cancer), making direct comparisons between patients difficult. Tumors often exhibit areas of poor differentiation and morphological changes, with cell scattering and spindle-shaped cells that are distinct from the bulk of the tumor, but pathologists do not routinely stain for markers of epithelial or mesenchymal phenotype as diagnostic and prognostic value is absent. Despite these shortcomings, histological examination of epithelial and mesenchymal markers in primary tumors and their corresponding metastases is important to determine whether EMT and MErT occurs clinically, with implications for the development of new approaches to cancer.

Recently, we have reported that breast and prostate cancer metastases express increased levels of E-cadherin when compared to the matched primary tumor [8, 9]. In addition, E-cadherin-negative MDA-MB-231 breast cancer cells were induced to re-express E-cadherin by in vitro coculture with liver parenchymal cells or following spontaneous metastasis to the lung in a mouse model [8]. However, despite the findings of E-cadherin re-expression and an accompanying morphological change, it remained to be seen whether a full or partial mesenchymal to epithelial transition had occurred. Thus, for the present study we evaluated the expression of mesenchymal and epithelial markers in a larger set of matched primary and metastatic tumor samples from breast cancer patients. We also focused on membranous expression of epithelial markers E-cadherin, β-catenin, connexin 26, and connexin43 as an indicator of normal function. Expression of epithelial markers was increased in metastases while expression of mesenchymal markers FSP1 and vimentin was variably changed, suggesting a partial MErT. In addition, we corroborated our results in a set of unmatched primary and metastatic prostate cancer samples and found that E-cadherin expression decreased with increasing metastatic tumor size, an observation that suggests that MErT is also reversible and helps to answer the question of whether metastases likely generate other metastases or if all metastases arise from the primary tumor.

Results

Breast Cancer Metastases Exhibit Increased Levels of Localization of Adherens Junction Components to the Membrane

A few studies have compared E-cadherin expression in the primary tumor and distant metastases [3, 9–11]. We recently reported on a small set of matched primary breast carcinomas and their metastases to the lung, liver, and brain [8]; besides bone, these comprise the most common sites of breast cancer metastases. In that study, we quantified both cytosolic and membranous staining as positive E-cadherin expression because E-cadherin expression was not always localized to the membrane. We have re-analyzed the data to include only positive membranous staining, as functional E-cadherin that both participates in intercellular adhesion and sequestration of the catenins is only localized at the membrane. In addition, we expanded the sample set to include additional pairs of matched specimens. Percentage of membrane expression was calculated as the number of cells positive for E-cadherin expression localized to the membrane over the total number of cancer cells in each field. Overall, 17/20 (85%) cases showed increased membranous E-cadherin expression in the metastases compared to the primary tumors (Fig. 1a), with this being consistent across the various sites; 2/2 (100%) of liver metastases, 5/6 (83%) of brain metastases, and 10/12 (83%) of lung metastases exhibited increased E-cadherin expression. The increase in E-cadherin levels across all tumor types was significant ($p<0.05$ by Wilcoxon paired analyses), as it was within brain and lung metastases (the liver numbers were insufficient for assessment).

Localization of β-catenin at the cell membrane has been shown to be a critical suppressor of cancer cell migration and invasion as it forms part of a stable adherens junction [12, 13]. We therefore evaluated primary and metastastic tumors for membranous β-catenin expression (Fig. 1b). Overall, 9/20 (45%) of metastases exhibited increased expression of membranous β-catenin; 7/12 (58%) of lung metastases, 1/2 (50%) of liver metastases, and 1/6 (17%) in brain metastases. When positive β-catenin expression is

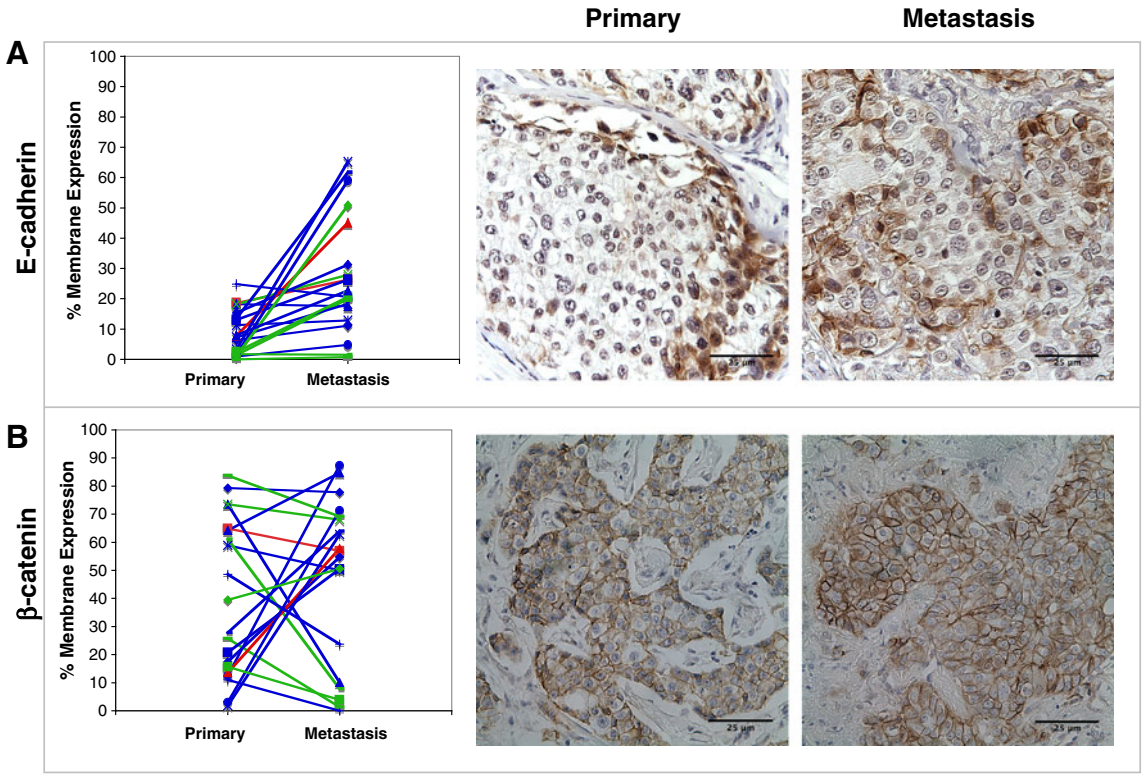

Fig. 1 Breast cancer metastases exhibit increased localization of adherens junctions components to the membrane. **a** Quantification of membrane-bound E-cadherin in breast cancer primary tumors and metastases. Representative images of a primary tumor exhibiting cytoplasmic or absent E-cadherin and the paired lung metastasis with membranous E-cadherin expression. *$p<0.05$ for all cases and in lung and brain metastases subsets. **b** Quantification of membranous β-catenin in primary and metastatic tumors. Images from a case that exhibited increased membranous β-catenin staining in a metastasis to the lung. Organ sites of metastases are color-coded: lung (blue), red (liver), and brain (green). Size bar in the photomicrographs is 25 microns

quantified as including both membranous and cytoplasmic expression, increased β-catenin is evident in metastases compared to primary tumors, in 9/12 (75%) of lung metastatases, 2/2 (100%) of liver metastases, and 1/6 (17%) of brain metastases (data not shown). None of these associations were statistically significant ($p<0.05$ for all cases; $p<0.20$ for lung; $p<0.10$ for brain), likely due to the high β-catenin in the primary site coupled with both EMT and MErT occurring on a spectrum, rather than absolute phenotype switches. Due to the activation of the downstream Wnt pathway, nuclear localization of β-catenin is most commonly associated with the invasive phenotype; therefore β-catenin involvement in an epithelial phentype maybe best be quantified by membranous and cytoplasmic localization.

Expression of Gap Junction Proteins is Increased in Breast Cancer Metastases to the Brain

While adherens junctions facilitate intercellular adhesion, gap junctions mediate intercellular communication by mediating the exchange of small molecules and ions through a membrane-spanning pore composed of connexins. In the breast, connexin 26 (Cx26) is expressed by luminal cells while connexin 43 (Cx43) is expressed by myoepithelial cells [14]. Loss of Cx26 and Cx43 has been shown to correlate with tumor progression in breast and colorectal cancer and over-expression of Cx43 reduces breast cancer metastasis [15–17]. Furthermore, just as re-expression of E-cadherin has been observed in metastases, increased expression of Cx26, Cx43, and Cx32 has been found in breast cancer lymph node metastases, suggesting that re-expression of gap junctions could also contribute to a MErT [18, 19]. We therefore surveyed the expression of membranous Cx26 and Cx43 in primary and metastatic tumors. For Cx26, 10/19 (53%) metastases showed increased membranous expression: 5/11 (45%) of lung metastases, 1/2 (50%) of liver metastases, and 4/6 (66%) of brain metastases (Fig. 2a). Increased expression of membranous Cx43 expression was observed in 55% (11/20) of all metastases, specifically in 4/12 (33%) of lung metastases, 1/2 (50%) of liver metastases, and 6/6 (100%) of brain metastases (Fig. 2b). For the most part, the two connexins changed, or stayed similar in parallel fashion within each metastasis. While there was

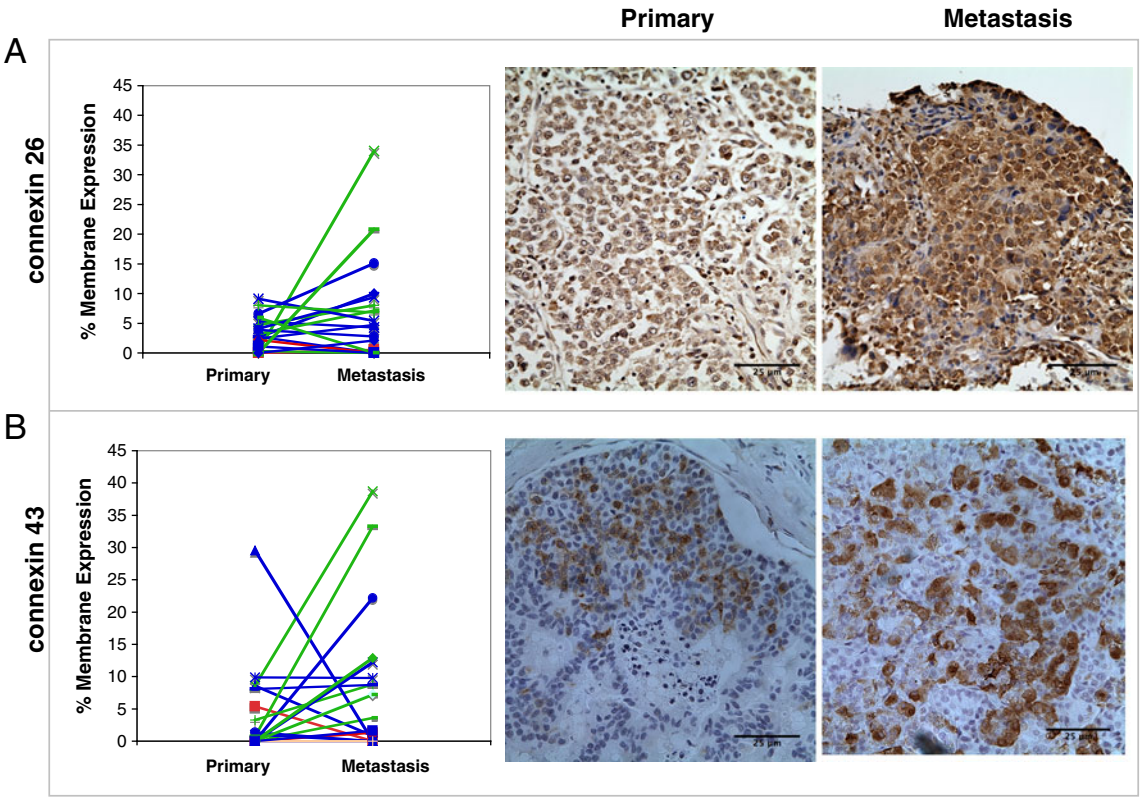

Fig. 2 Expression of gap junctional proteins is increased in breast cancer metastases to the brain. Quantification of membranous Cx26 (**a**) and Cx43 (**b**) staining in primary and metastatic breast cancer tumors. Shown are representative images of connexin staining in primary tumors and brain metastases. Organ sites of metastases are color-coded: lung (blue), red (liver), and brain (green). Size bar in the photomicrographs is 25 microns. *$p<0.05$ for brain subsets of Cx26 and Cx43

no correlation in metastases to lung or liver, both Cx26 and Cx43 expression was strikingly increased in metastases to the brain ($p<0.05$) but the differences in connexin 26 or 43 expression between primaries and metastases when all cases were considered together did not reach statistical significance ($p<0.20$ for Cx26 and $p<0.10$ for Cx43). This was because there was no correlation in cases involving metastases to the lung ($p<0.50$ for Cx26 and $p<0.80$ for Cx43).

Persistence of Mesenchymal Markers in Metastases Suggests a Partial Mesenchymal to Epithelial Reverting Transition

To determine if the increase in epithelial markers signified the occurrence of a full MErT, which includes a loss or decrease in expression of mesenchymal markers in metastases, we next evaluated the expression of FSP1 and vimentin. FSP1 is considered one of the few truly fibroblast-specific markers and is commonly used as an early marker of EMT [20, 21]. Vimentin is also a widely accepted marker of the mesenchymal phenotype in EMT. During EMT, cells undergo a shift from using cytokeratin intermediate filaments to vimentin intermediate filaments, which are involved in the changes in adhesion and motility [22, 23]. Immunohistochemistry revealed that overall only 9/19 (47%) of metastases showed decreased expression of FSP1: 4/11 (36%) of lung metastases, 1/2 (50%) of liver metastases, and 4/6 (66%) of brain metastases (Fig. 3a). Similarly, 13/20 (65%) of metastases exhibited decreased expression of vimentin: 7/12 (64%) of lung metastases, 2/2 (100%) of liver metastases and 4/6 (66%) of brain metastases (Fig. 3b). For metastases that did display a decrease in expression of FSP1 or vimentin, the degree of change was small relative to the change observed in E-cadherin. Statistical analyses revealed no statistically significant difference between FSP1 ($p>0.80$ for all cases; $p>0.50$ for cases metastatic to lung; $p>0.50$ for cases metastatic to brain) or vimentin ($p>0.15$ for all cases; $p>0.45$ for cases metastatic to lung; $p<0.60$ for cases metastatic to brain) expression between primary and metastatic tumors. The lack of a dramatic downregulation of mesenchymal markers, along with the variability of epithelial markers noted above, suggest that only a partial MErT occurs during metastatic colonization.

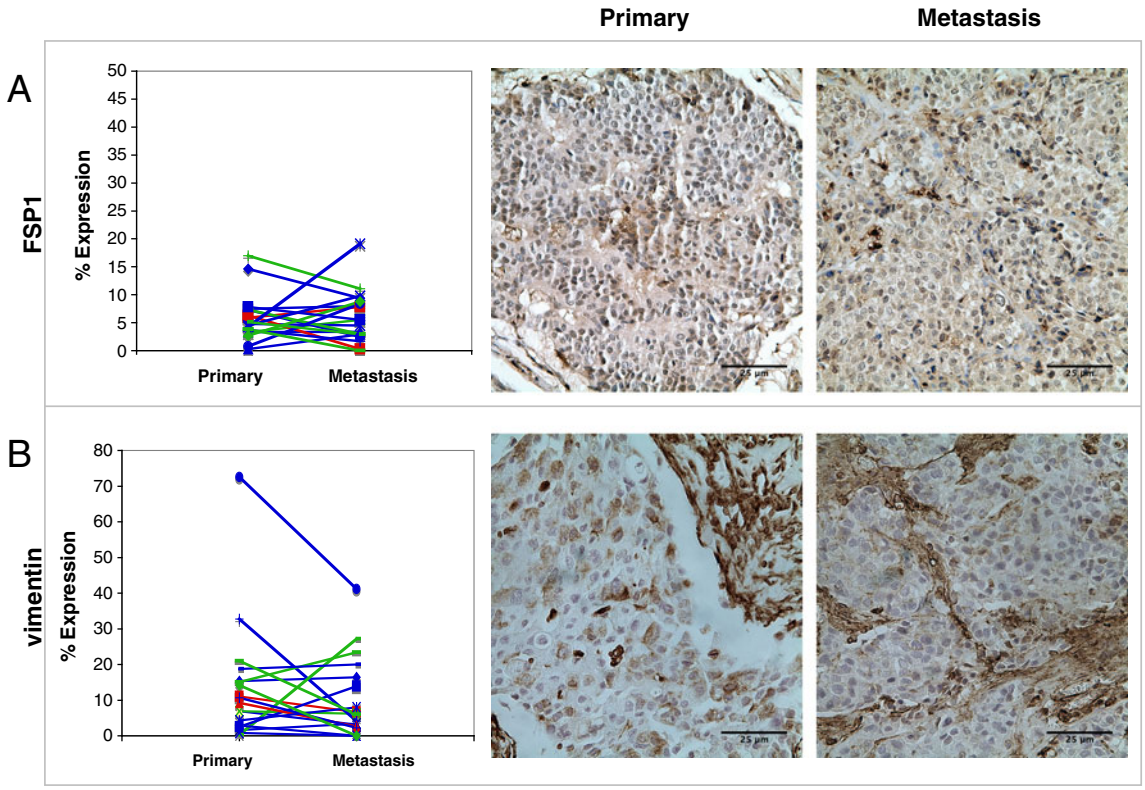

Fig. 3 Mesenchymal markers persist in metastases, suggesting only a partial MErT. Quantification of immunostaining for mesenchymal markers FSP1 (**a**) and vimentin (**b**). Images of FSP1 and vimentin staining in primary tumors and metastases. Organ sites of metastases are color-coded: lung (blue), red (liver), and brain (green). Size bar in the photomicrographs is 25 microns

E-Cadherin Expression is Inversely Correlated with Size of Metastasis

To extend our findings beyond breast cancer metastases, we obtained a number of unmatched prostate carcinoma primary tumors and metastases. Organ sites of metastases included liver, lung, kidney, and thyroid. Primary and metastatic tumors were immunostained for membrane-associated E-cadherin and the percentage of cancer cells staining positive for E-cadherin was quantified in each field. Metastases exhibited increased staining of E-cadherin compared to primary tumors ($p<0.05$), suggesting that E-cadherin re-expression can occur in other cancers besides breast carcinoma (Fig. 4a). Due to a shortage of specimens, staining for other epithelial and mesenchymal markers was not performed.

Several of the metastatic specimens from individual patients contained multiple foci of different sizes. The metastatic foci within one patient sample were divided into three categories based on size: less than 50 μm in diameter (small), between 50 μm and 100 μm in diameter (medium), and larger than 100 μm in diameter (large) (Fig. 4b). It is recognized that sizing of tumor nodules depends on selection, but as our earlier study found an inverse correlation between E-cadherin levels and distance from normal parenchyma, the cross-sectional area was considered more critical than the absolute volume. The staining intensity of E-cadherin was quantified for each individual focus. Interestingly, E-cadherin expression inversely correlated with tumor size, with increased E-cadherin expression in small metastases compared to large ($p<0.01$) (Fig. 4c), suggesting that the partial MErT that allows for metastatic colonization is transient and reversible like the EMT that enables metastatic dissemination.

Discussion

One of the major limitations of studying metastasis in vivo is that studies involving animal models and clinical samples are end-stage time points that can only provide a snapshot of the metastatic cascade at the point of tissue harvest. Although intravital imaging and use of organotypic bio-reactors has improved the ability to visualize metastasis at various stages, the phenotypic plasticity exhibited during EMT and MErT is nonetheless difficult to capture [24–26]. Evidence of EMT and MErT in clinical specimens is rare and has been used as an argument that cancer-associated

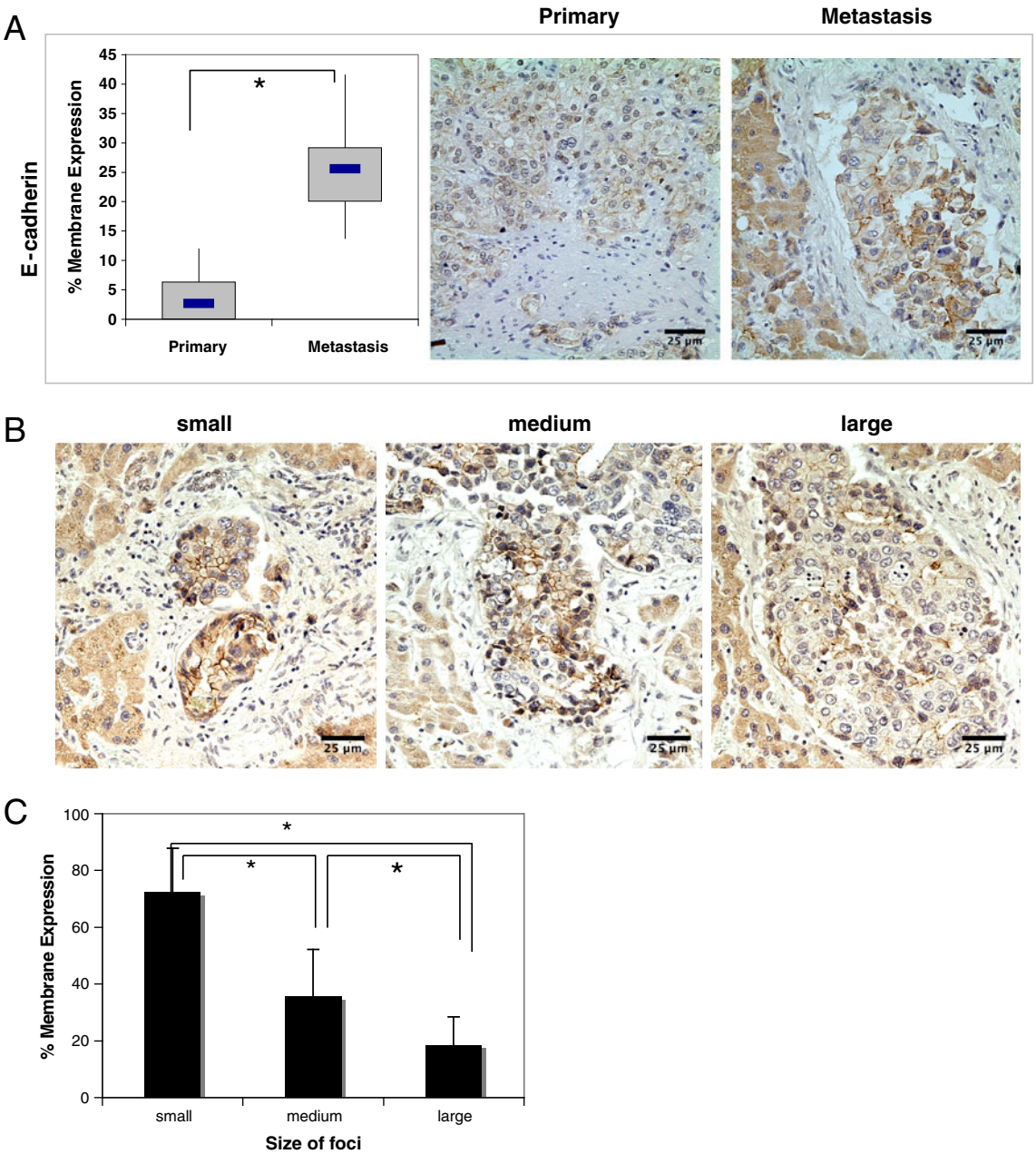

Fig. 4 E-cadherin expression in prostate cancer metastases is inversely correlated with size of metastasis. **a** Quantification and representative images of prostate cancer primary and metastatic tumors immunostained for E-cadherin. *$p<0.05$ Images of three random fields were quantified with ImageJ. **b** Images of metastatic tumors stained for E-cadherin as categorized by size: small (less than 50 μm in diameter), medium (between 50 μm and 100 μm) and large (bigger than 100 μm). **c** Quantification of E-cadherin expression in different sized prostate cancer metastases. *$p<0.05$. Size bar in the photomicrographs is 25 microns

EMT does not occur during the course of disease. Using matched primary and metastatic tumors, we have examined expression of epithelial and mesenchymal markers in specimens obtained from human breast cancer patients. Unfortunately, such paired specimens are few due to advances in imaging obviating the necessity for subsequent surgical biopsies, limiting the ability to accrue sufficient numbers that would allow for rigorous statistical analyses and subset identifications. However, within this limitation, our results show that the occurrence of cancer-associated EMT and MErT is possible (Fig. 5).

Paget's "seed and soil" hypothesis posits that cancer cells can only survive and grow in appropriate environments; the reversible phenotypic plasticity of cancer cells during EMT and MErT is therefore one way in which cancer cells can adapt to the foreign soil of ectopic organ microenviron-

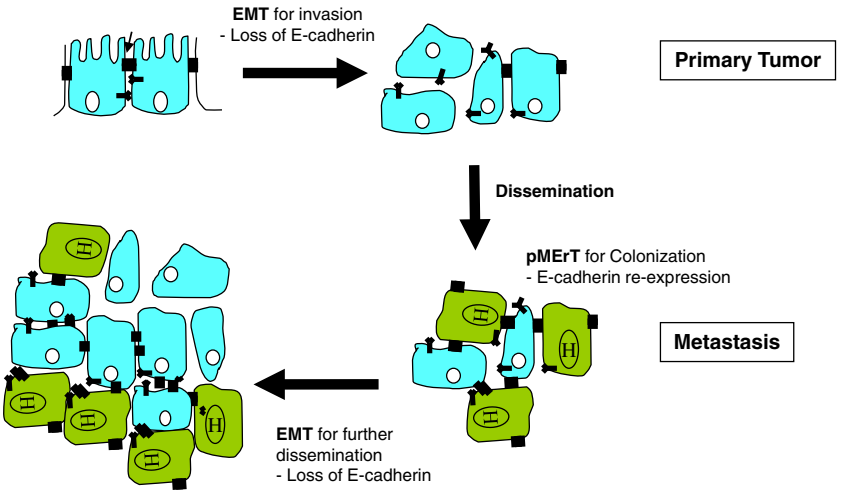

Fig. 5 Model of reversible phenotypic transitions during metastasis. EMT and loss of E-cadherin enables dissemination, followed by E-cadherin re-expression and a partial MErT that facilitates metastatic colonization at a secondary site. MErT is reversible, and with tumor growth may undergo an additional EMT

ments. Expression of adhesion molecules has been shown to be necessary to complete the final steps of the metastatic cascade including intravasation and colonization [27]. Based on previous observations of increased E-cadherin expression in metastases compared to primary tumors, we expanded our analysis to include E-cadherin binding partner β-catenin, gap junction molecules Cx26 and Cx43 and mesenchymal markers FSP1 and vimentin to discern whether a full or partial MErT occurs (summarized in Table 1). We limited our quantification of E-cadherin, β-catenin, Cx26, and Cx43 to expression localized to the membrane to account for proteins functioning in the epithelial phenotype, as dysfunctional proteins are commonly dislocated in the cytoplasm or nucleus during tumor progression. Increased expression of membranous E-cadherin was observed in metastases compared to primaries, across all organ sites of metastases. While we expected these results in metastases to lung and to liver where E-cadherin is expressed by pneumocytes and hepatocytes, it was surprising that 83% of metastases to the brain also exhibited increased E-cadherin expression. Of interest, disseminated cells in lymph nodes do not show similar E-cadherin expression but more closely resemble the original primaries [28]. Breast cancer cells that metastasize to bone have been shown to express OB-cadherin, the cadherin expressed by osteocytes, so it was expected that metastases would exhibit increased expression of the adhesion molecule native to the ectopic organ [29, 30]. Thus, increased E-cadherin expression was not expected in metastases to the brain, which primarily expresses N-cadherin. When we queried N-cadherin expression in primay and metastastic tumors, only 2/5 brain metastases exhibited increased N-cadherin expression (data not shown).

It is not surprising that an overall corresponding increase in membranous β-catenin was not observed in metastases, as in all specimens the percentage of cells expressing β-catenin was higher than the E-cadherin-expressing cells. Thus, there was limited amount of increase that could be noted with β-catenin. This high level could be due to β-catenin binding to other cadherins. E-cadherin is not the only molecule that sequesters β-catenin, as the cytoplasmic domains are conserved among the type I classical cadherins. To test this, samples were also stained for N-cadherin (data not shown). While there was no consistent pattern of N-cadherin expression between primary tumors and metastases, high N-cadherin expression in the primary tumor was observed in many cases that exhibited no change or decreased localized β-catenin expression in metastases.

We also evaluated expression of gap junction molecules as another measure of epithelial gene expression in MErT. Cx26 and Cx43 are disparately expressed in the breast-luminal cells express Cx26 while myoepithelial cells express Cx43 [14]. Although the luminal and basal breast cancer subtypes arise from these two different cell types, there was no association between connexin expression and ER/PR/Her2 status, and therefore breast cancer subtype (Table 1). Overall, metastases exhibited increased expression of Cx26 and Cx43 compared to the primary tumors. This was most striking in brain metastases, where 66% of brain metastases demonstrated increased Cx26 expression and 100% showed increased Cx43 expression. In the brain, Cx26 and Cx43 are expressed by astrocytes, which suggests that gap junctions and not adherens junctions may be the driving force behind brain metastases. We have hypothesized that MErT in metastatic colonization serves to protect the metastatic cancer cell from inflammatory or chemotherapeutic insult [4]. Recent in vitro work by the Fidler group supports both our findings of increased connexin expression in brain metastases and also the theory that this re-expression confers a survival advantage. Melanoma or breast cancer cells

Table 1 Summary of epithelial and mesenchymal marker expression data. Green, cases that exhibited an increased expression in metastases; red, decreased expression in metastases compared to primary tumors; yellow, absent or no change in expression; white, unable to quantify sample

	Case	Subtype		E-cad	β-cat	Cx26	Cx43	FSP1	Vim
LUNG	1	ER-/PR-/HER2-	Basal						
	4	ER+/PR+/HER2-	Luminal						
	8								
	9	ER-/PR-/HER2-	Basal						
	10	ER-/PR-/HER2+	HER2						
	13	ER+/PR-/HER2-	Luminal						
	14								
	16	ER-/PR-/HER2-	Basal						
	18	ER-/PR-/HER2+	HER2						
	19	ER-/PR-/HER2-	Basal						
	20	ER-/PR+/HER2+	HER2						
	21								
	26	ER+/PR-/HER2-	Luminal						
				10/12 (83%)	7/12 (58%)	5/11 (45%)	4/12 (33%)	4/11 (36%)	7/12 (58%)
LIVER	3	ER-/PR-/HER2-	Basal						
	15	ER-/PR-/HER2+	HER2						
				2/2 (100%)	1/2 (50%)	1/2 (50%)	1/2 (50%)	1/2 (50%)	2/2 (100%)
BRAIN	4	ER+/PR+/HER2-	Luminal						
	11	ER+/PR-/HER2-	Luminal						
	12	ER-/PR-/HER2+	HER2						
	22								
	24	ER-/PR-/HER2+	HER2						
	25								
				5/6 (83%)	1/6 (17%)	4/6 (66%)	6/6 (100%)	4/6 (66%)	4/6 (66%)

UP
DOWN
NO CHANGE
NO SAMPLE

cultured with astrocytes demonstrated reduced chemosensitivity, which was mediated by expression of connexins [31–33].

When immunostaining was performed for FSP1 and vimentin, expression of these markers in metastases was either unchanged or slightly decreased, suggesting only a partial MErT. Limitations in tissue prevented us for more examining other markers of mesenchymal phenotype, particularly N-cadherin which promotes interactions during intravasation and extravasation [34]. However, in a limited sampling, we did not find a correlation between N-cadherin levels and metastases, though this may reflect the metachronicity between the carcinoma dissemination and specimen acquisition. In addition, tumors are typically surrounded by reactive fibrosis and normal stromal cells that stain positive for mesenchymal markers so the possibility of false positives is high. Ideally, dual staining for breast cancer-specific and mesenchymal markers would overcome this problem; however, a reliable breast cancer-specific marker does not exist. Cell-cell adhesion and cell motility are usually viewed as opposite sides of the epithelial and mesenchymal phenotypic spectrum. However, partial EMT and MErT in which cells maintain some level of both is not an unusual phenomenon, as many examples can be found throughout cancer progression. During invasion, tumors have been shown to invade the ECM collectively as strands of cancer cells that maintain expression of adhesion molecules [35]. Similarly, during extravasation cancer cells re-express molecules that permit adhesion to endothelial cells yet still maintain the ability for transendothelial migration [36, 37].

Finally, we also found that E-cadherin expression decreases with increasing metastatic tumor cross-sectional area, if not actual size, suggesting that just as EMT is reversible, so is MErT. These data support earlier experimental evidence that the EMT that allows for escape from the primary lesion is not fixed but can be reverted during metastatic seeding [1, 8, 9]. However, often pathological examination of large metastases removed for palliative or diagnostic needs present de-differentiated cells reminiscent of the original EMT, which superficially appears at odds with our model of MErT. These data can be reconciled by our analysis of the prostate carcinoma micrometastases. In evaluating expression of E-cadherin based on metastasis size, we found the larger metastases (all still microscopic clinically) were less likely to express E-cadherin at the membrane, implying a re-emergence of EMT as with tumor growth. Thus, the phenotypic plasticity of carcinomas allows for continual repositioning of the tumor cell to provide a survival or dissemination advantage.

Evident in the stainings is a heterogeneity of markers within a given tumor at both the primary and metastatic sites. This has always been taken as evidence of the well-accepted concept of carcinoma progression, and often goes unremarked. However, this phenotypic diversity does allow for the possibility that the more epithelial cells in the primary actually seed and give rise to metastases [38, 39]; this is a postulate that cannot be tested by human observational reports even in the absence of markers in a

noted primary due to the metachronosity of the dissemination from the specimen acquisition. We suggest that this does not represent the majority of the epithelial-like metastases based on our experimental findings that demonstrate that a xenograft derived from clonal human breast or prostate (or colorectal, data not published) cancer cells lacking E-cadherin expression can form E-cadherin-expressing metastases [8]. Additionally, we reported that primary human carcinoma cells can be 'educated' to re-express epithelial markers by hepatocytes. Thus, we undertook this study to determine whether the human situation was consistent with such a EMT/MErT hypothesis. The correlations herein support this possibility. Additionally, the seeming evolution of the metastases towards a more mesenchymal-like state with increasing size (Fig. 5) and distance of the carcinoma cells from normal parenchymal [8], suggests the metastable nature of the phenotype displayed and supports the model.

The reversibility of MErT at the secondary site alludes to the question of whether all metastases necessarily arise from the primary tumor or whether metastases can give rise to metastases. An autopsy study of breast cancer patients found that the frequency of metastases to non-common sites was lower when metastases to the lung, liver, or bone were not already present [40]. It has been shown in a mouse model that systemic metastases arise in mice with large lung metastases in the absence of the primary tumor and also shown in melanoma dissemination to lung [41, 42]. One explanation is dormant cells were already seeded in the lung prior to primary tumor removal, but parabiosis experiments showed that the non-tumor bearing partner could develop metastases [43]. Despite these observations, the mechanism by which these secondary metastases occur is unknown. Here we suggest that EMT may occur following MErT in the metastatic site to engender these secondary metastases. Ultimately, the persistence of mesenchymal characteristics in MErT, despite the re-expression of epithelial genes and adhesion molecules, enables metastatic cancer cells to adeptly adapt to changing environments—from primary tumor to secondary organ and beyond.

Materials and Methods

Immunohistochemistry

All studies were performed on de-identified specimens obtained during clinically-indicated procedures; these were deemed to be exempted (4e) from human studies by the University of Pittsburgh Institutional Review Board.

Paraffin-embedded patient samples, excess to clinical need, were obtained from the University of Pittsburgh Tissue Banks, primarily coming from Magee Womens Hospital of UPMC and UPMC Shadyside Hospital, under informed consent of patients undergoing diagnostic and therapeutic procedures. Sections underwent antigen retrieval in citrate solution (Dako) and were incubated with primary antibodies: E-cadherin (Cell Signaling), β-catenin (abcam), connexin 26 (abcam), connexin 43 (abcam), FSP1/S100A4 (abcam), and vimentin (abcam) followed by biotin-conjugated secondary antibody (Jackson Laboratories). Antigen staining was performed using DAB (Vector Laboratories) then counterstained with Mayer's hematoxylin. Secondary antibody alone served as a negative control and adjacent normal tissue served as an internal positive control. Images of three randomly-selected microscope fields of each sample were taken and the percentage of cancer cells with positive staining was quantified as the number of positive cells over the total number of cancer cells in that image. Microscope fields shown were selected to account for the heterogeneity of each sample.

Statistical Analysis

For the matched breast cancer primary and metastatic tumor samples, statistical significance was calculated using the two-tailed Wilcoxon matched pairs test. P values were calculated for all cases combined as well as for individual organ sites. For the unmatched prostate cancer samples the Mann–Whitney test was used. For comparing E-cadherin expression between foci of different sizes, the Kruskal-Wallis ANOVA test was used.

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Mesenchymal–epithelial transition (MET) as a mechanism for metastatic colonisation in breast cancer

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Abstract As yet, there is no cure for metastatic breast cancer. Historically, considerable research effort has been concentrated on understanding the *processes* of metastasis, how a primary tumour locally invades and systemically disseminates using the phenotypic switching mechanism of epithelial to mesenchymal transition (EMT); however, much less is understood about how metastases are then *formed*. Breast cancer metastases often look (and may even function) as 'normal' breast tissue, a bizarre observation against the backdrop of the organ structure of the lung, liver, bone or brain. Mesenchymal to epithelial transition (MET), the opposite of EMT, has been proposed as a mechanism for establishment of the metastatic neoplasm, leading to questions such as: Can MET be clearly demonstrated *in vivo*? What factors cause this phenotypic switch within the cancer cell? Are these signals/factors derived from the metastatic site (soil) or expressed by the cancer cells themselves (seed)? How do the cancer cells then grow into a detectable secondary tumour and further disseminate? And finally—Can we design and develop therapies that may combat this dissemination switch? This review aims to address these important questions by evaluating long-standing paradigms and novel emerging concepts in the field of epithelial mesencyhmal plasticity.

Keywords EMT · MET · Mesenchymal · Epithelial · Transition · Breast cancer · Metastasis · Proliferation

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1 Overview

Breast cancer is the most common malignant tumour diagnosed among women worldwide [1, 2]. It is also the second leading cause of cancer-related deaths in women. In these patients, more than 90 % of breast cancer-related death is caused not by the primary tumour, but by their metastases at distant sites. As a result of early diagnosis by screening, improved surgical techniques and implementation of adjuvant therapies, there is a general downward trend in the prevalence of disseminated disease in breast cancer patients [3]. Although local radiation therapy and systemic adjuvant therapy reduce the incidence of metastasis by eliminating the breast cancer cells that have disseminated at the time of diagnosis, their effectiveness is far from guaranteed. More than 80 % of patients receive systemic adjuvant therapy together with the initial local surgical treatment, once diagnosed with breast cancer. An estimate of the benefit of adjuvant therapy even in the most medically advanced treatment centres is a reduction in the annual odds of death ranging from 8 to 28 % [4] and a reduction in the 10-year survival rate by less than a third [5, 6]. Clearly, many people are treated with these debilitating therapies to no avail.

2 The process of metastasis via EMT

Although uncontrolled epithelial proliferation and angiogenesis [7] are major facilitating mechanisms in metastasis, additional processes are needed for the successful establishment of a metastatic tumour [8, 9]. Early in the metastatic cascade (Fig. 1), cancer cells from the primary tumour acquire invasive properties and gain access to the blood or lymphatic vascular systems, which is aided by neo-angiogenesis and remodelling/destruction of the basement membrane [10–12]. In the bloodstream (and presumably in lymphatic vessels), intravasated circulating tumour cells (CTCs) are capable of surviving and eventually reach 'hospitable' distant secondary sites, such as bone, lungs, brain and liver. Extravasation of CTCs at the secondary site requires recognition of and adhesion to vascular endothelial cells followed by matrix degradation [9, 13, 14]. Finally, the circulating tumour cells must invade the secondary tissue to become disseminated tumour cells (DTCs), typically studied in the bone marrow. All of these processes are evidence of a more motile and plastic 'mesenchymal-like' phenotype that promotes movement from a syncytial mass and invasion through tissue [15].

The fate of DTCs at the ectopic site varies, although the vast majority of these cells do not survive even a week under experimental conditions [16, 17]. The surviving cells could remain indolent as isolated DTCs or as small micrometastases. Alternatively, some DTCs could re-establish colonies to give rise to clinically overt, macroscopic secondary tumours—metastases. A daunting issue for breast cancer is the propensity for subclinical metastases to lie undetected, presumably dormant, for even over a decade before emerging.

The precise mechanisms that are involved in the transition of the subset of non-invasive tumour cells into cells with metastatic potential are still not well understood. However, accumulating evidence suggests that an epithelial to mesenchymal transition (EMT)-like process, first described in embryonic development, is one of the main mechanisms involved in breast cancer metastasis and most likely contributes to metastases from all types of carcinomas. Somewhat consistent with its role in normal mammary gland development [18, 19], EMT has also been shown to be responsible for converting a fraction of non-invasive tumour cells in a solid tumour into cells with the ability to invade the basement membrane, intravasate and survive in the circulation, and extravasate at a distant secondary site (reviewed in [20–22]).

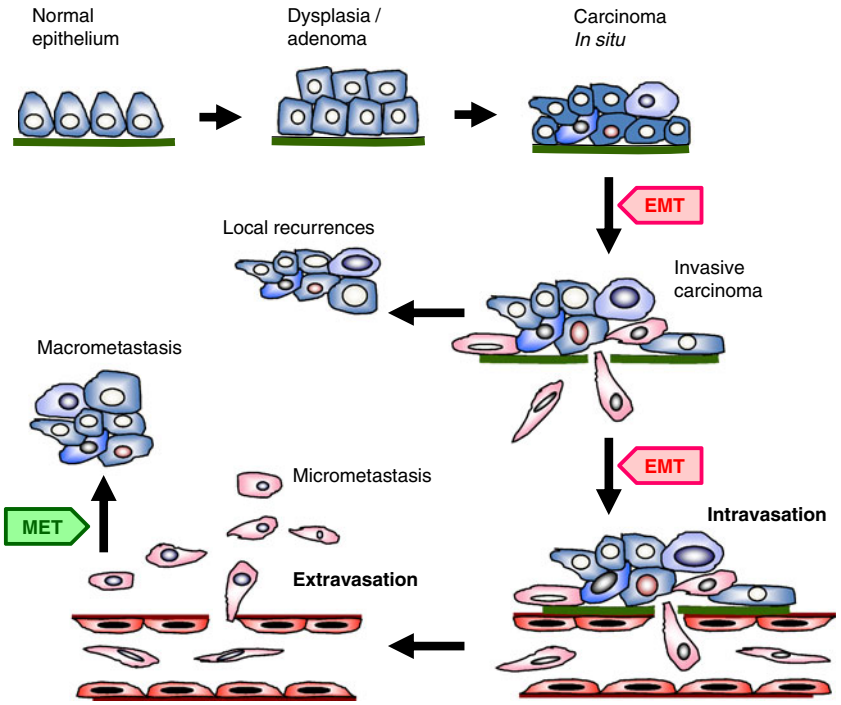

Fig. 1 The illustration elaborates the sequential EMT and MET events that are hypothesised to take place in breast cancer progression. Normal epithelial cells undergo a series of transformational changes to become malignant. Clonal proliferation of malignant cells gives rise to invasive carcinoma. Some of these cells invade local tissues to form local recurrences while another fraction of cells undergoes EMT and intravasates into the neighbouring blood vessels. These intravasated cells may remain in the circulation as CTCs or extravasate at a distant site. The extravasated tumour cells may remain indolent as DTCs or micrometastasis (micromets) or form macrometastasis (MacroMets) by a reverse mechanism, MET. The illustration is an adaptation from Thiery et al. [102]

3 Secondary tumour formation via MET

The inefficiency of metastatic establishment has necessitated a search for an underlying mechanism that would provide for the key attributes of cell survival in an ectopic environment. A process opposite to the initial EMT at the primary tumour site, mesenchymal to epithelial transition (MET), is an evolving and relatively under-investigated mechanism that is considered to contribute substantially to the colonisation of DTCs into metastatic tumours at the secondary site [23–26]. Recently, this well accepted 'late metastasis' concept was challenged by certain groups demonstrating that dissemination of tumour cells occurs at an early stage of primary tumour establishment [27, 28]. This 'early metastasis' paradigm suggests that a fraction of primary tumour cells comprising stem cell-like characteristics with high CD44 and low CD24 ($CD44^{high}/CD24^{low}$) have the potential to depart the primary tumour site relatively early and form metastatic colonies at distant sites [29]. The $CD44^{high}/CD24^{low}$ phenotype in breast cancer cell lines has been linked to EMT through the mesenchymal attributes of breast cancer stem cells, which also have dramatically enhanced malignant properties [30, 31]. In either case, the disseminated cells appear to be of a mesenchymal phenotype, which is at odds with the finding of epithelial-like breast cancer nodules in the ectopic tissues [32–34].

Given the emphasis on EMT and metastatic potential, it was quite surprising that Korpal et al. [35] found recently that elevated levels of the epithelial microRNA (miR)-200 family in primary breast tumours already predisposed the cancer to successful metastasis, as evidenced in poorer outcomes. The miR-200 family members have been shown to promote E-cadherin re-expression via the repression of ZEB family genes and vice versa, and this mechanism has been implicated in cancer invasion and metastasis [36–40]. Indeed, several 'epithelial' miRs have been implicated in promoting metastatic colonisation (reviewed in [41]), supporting a role for MET. Recently, metastatic competence in prostatic and bladder carcinoma systems was very clearly related to epithelial variants of established cell lines, rather than mesenchymal, and linked to expression of pluripotency and self-renewal gene expression [42]. Induction of EMT in each cell system by Snail overexpression quashed the expression of these genes, reduced tumourigenicity and abrogated metastatic potential. These recent studies are in sharp contrast to many studies illustrating the pro-metastatic role of EMT.

Given that epidemiological studies show that death from metastases is responsible for 90 % of all human cancer-related mortalities [43] and that a majority of breast cancer deaths are due to metastases rather than primary tumours [44–46], a closer examination of MET as a potential mechanism contributing to the formation of secondary breast tumours is of paramount importance and hence the focus of this review.

4 E-cadherin expression in primary and secondary breast tumours

One of the key strategies in addressing this question is the assessment of the archtypical epithelial cadherin E-cadherin in metastases. These studies have been limited because, typically, metastases are not resected, and thus the tissue is not available to study. Of the many EMT-related molecules, the most widely studied is the intercellular adherence protein E-cadherin (CDH1), which is currently thought to be a suppressor of tumour invasion [47–49]. The functional loss or downregulation of E-cadherin from epithelial cells is considered a hallmark of EMT [50, 51]. Kowalski et al. have reported distant metastases expressing an equal or stronger E-cadherin signal than the respective primary tumours from which they originated [32]. They saw all metastatic tumours of invasive ductal carcinoma re-expressing E-cadherin irrespective of the E-cadherin status of the primary tumours. Although not investigated, they suggested that both translational and post-translational regulation of E-cadherin take place at the metastatic site in order to facilitate the establishment of secondary tumour colonies. In another study, Saha et al. reported re-expression of E-cadherin in bone metastasis originating from E-cadherin-negative poorly differentiated primary breast carcinoma [52]. In a more recent study, Chao and colleagues reported the re-expression of E-cadherin at distant metastatic tumours arising from E-cadherin-low or E-cadherin-negative primary tumours [23]. They reported strong E-cadherin expression in more than 50 % of liver, brain and lung metastasis originating from infiltrating ductal carcinoma of the breast [34].

Still, there have been questions as to whether the few extant metastases arise from tumour cells that have undergone EMT or rare disseminated tumour cells retaining the epithelial phenotype [53]. This cannot be addressed by examining human tumour specimens as all primary tumours demonstrate phenotypic heterogeneity, and the ontogeny of the metastases can only be indirectly inferred. Experimental approaches have instead begun to answer this question. First, E-cadherin downregulation was found necessary to initiate an invasion/dissemination-type response in tumour spheroid models [54]. More to the point, a recent paper demonstrated that the initial spontaneous lung micrometastases from xenografts of the invasive, metastatic and mesenchymal-like MDA-MB-231 human breast cancer cell line all present re-expression of E-cadherin [23]. These studies thus provide proof of principle that the metastatic cascade invokes E-cadherin emergence and thus supports a MET-like phenomenon.

5 MET in MDA-MB-468 xenograft local lymphovascular invasion

Our recent work has shed light on the need for MET in successful seeding and outgrowth of metastases from the primary site. The extant model system used a phenotypically plastic breast cancer line that responds to known tumour microenvironmental cues. The MDA-MB-468 breast cancer cell line has a modal chromosome number of 35 and was derived from a 51-year-old woman with a pleural effusion in 1977 [55]. It has a doubling time of 2.5–3 days [56] and demonstrates a slow migratory activity *in vitro* suggesting a low level of invasiveness [57–59]. The cells are predominantly epithelial and express E-cadherin but are deficient in α-catenin [60] and lack some epithelial markers such as ZO-1 [61]. Previous studies have used this cell line as a model for *in vitro* EMT after treatment with epidermal growth factor (EGF) and hypoxia [62–64]. Recently, this was extended to *in vivo* studies [65], where regional EMT could be demonstrated in the primary tumour, was evident in the CTCs by RT-PCR, and in blood vessels of both the primary tumour and lung metastases by immunohistochemistry.

Our own pilot studies have confirmed the MDA-MB-468 as a suitable model for *in vivo* EMT experimentation and analysis of MET. Analysis of MDA-MB-468 xenografts (Fig. 2) revealed that these tumours were regionally positive for vimentin *and* E-cadherin, suggesting a tumour with a so-called metastable phenotype [66, 67], a situation also noted in human breast tumour micrometastases [34]. However in some regions of the tumours, as indicated with the arrow in Fig. 2b, the cells at the invasive front appeared to be arranged in thin rows in 'Indian file' formation, interspersed among the stromal connective tissue. These invading cells stained positive for vimentin and negative for E-cadherin, consistent with an EMT. This 'Indian file' appearance is typical of lobular carcinoma of the breast [68, 69], where E-cadherin loss is common [70].

Invasion of tumour cells into the neighbouring lymphovasculature was observed in association with many of the these MDA-MB-468 tumours. Two different forms of lymphovascular invasion (LVI) were observed. The majority of lymphovascular-invaded tumour cells existed as large tumour emboli, although scattered individual cells were also occasionally seen within extra-tumoural lymphovasculature. The tumour emboli consisted of tightly cohesive and considerably larger tumour cell clusters. The tumour emboli expressed E-cadherin to an extent that was noticeably higher than in the primary tumour and also stained for cytokeratin (not shown), further confirming their epithelial nature. These observations led to speculation about the *precise* nature of the invading cells, whether these invaded cells had been mesenchymal at the time of invasion and later converted into epithelial phenotype within the vasculature,

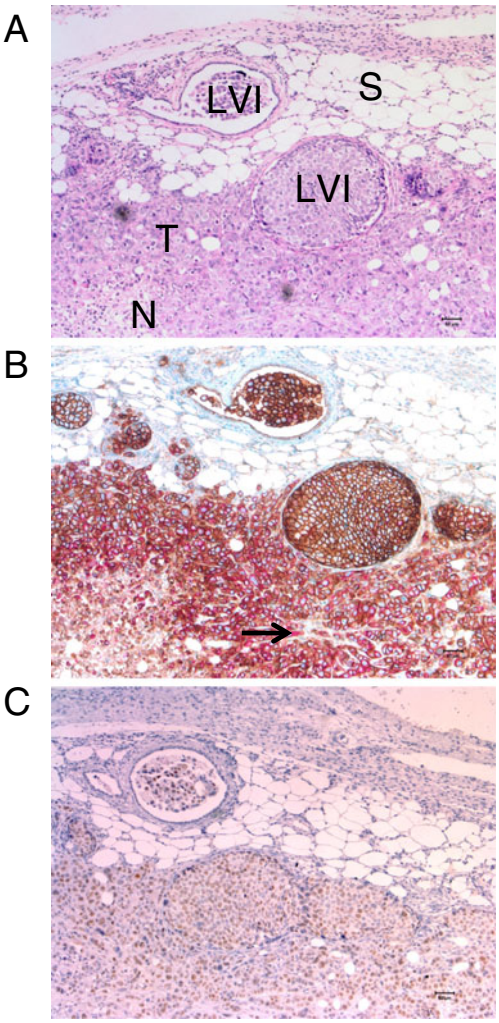

Fig. 2 Evidence of MET in local spread (lymphovascular invasion) of MDA-MB-468 primary xenograft tumours. **a** Haematoxylin and eosin staining indicating regions of tumour (T), peripheral stroma (S) necrosis (N) and locally metastasized tumour in the lymphovasculature (LVI); **b** to examine the EMP status, double IHC of E-cadherin and vimentin was performed using rabbit monoclonal anti-E-cadherin (clone EP700Y, Abcam, UK) and mouse monoclonal anti-vimentin (clone V9, DakoCytomation, Denmark) primary antibodies at dilutions of 1:500 and 1:100, respectively. The IHC procedure was carried out in an autostainer (BenchMark ® ULTRA, Ventana Medical Systems, Inc., USA). E-cadherin is indicated as brown colour and vimentin stained red, detected using UltraView Universal DAB (Ventana Medical Systems, Inc., USA) and UltraView Universal Alkaline Phosphatase Fast Red (Ventana Medical Systems, Inc., USA), respectively. **c** Ki-67 staining. All images were taken at a magnification of ×200; *scale bar*=50 μM

or were epithelial even at the time of invasion. However, in support of the former scenario, we witnessed in some LVIs a gradual transition of invaded tumour cells from mesenchymal to metastable and then to the epithelial phenotype, indicating the existence of a MET process (Fig. 3).

These observations are consistent with the literature suggesting the occurrence of MET at a distant metastatic site

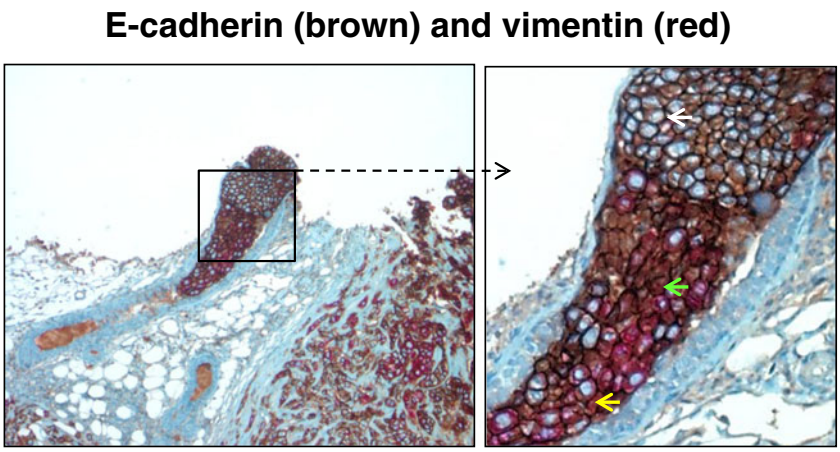

Fig. 3 Spectrum of MET and EMT seen in tumour emboli. A gradual transition from mesenchymal to epithelial status was observed in some established tumour emboli found within local lymphovascular spaces. These emboli consisted of regions of vimentin-expressing mesenchymal cells (*yellow arrow*), both E-cadherin- and vimentin-expressing 'metastable' cells (*green arrow*) and predominantly E-cadherin-expressing epithelial cells (*white arrow*). Double IHC of E-cadherin and vimentin was performed as above (Fig. 2 legend). E-cadherin is indicated as *brown* and vimentin stained *red*

during the formation of secondary tumours in breast cancer [23, 25, 32, 52]. However, our work suggests the contribution of MET as an early event in the metastatic process. Oltean et al. [71, 72] used FGF receptor reporter constructs to illustrate considerable plasticity in primary Dunning rat prostatic adenocarcinoma cells, and Tsuji et al. [73] and Martorana et al. [74] have illustrated cooperativity between epithelial and mesenchymal components in hamster oral squamous cell carcinoma and rat mammary carcinoma cellular systems, respectively. Indeed, the work of Tsuji and colleagues suggest a cooperativity model rather than plasticity *per se*, since their mesenchymal cells had an increased invasive but decreased metastatic phenotype, whereas their epithelial counterparts established lung metastases. They hypothesised that the EMT cells are responsible for leading the invasion and intravasation of epithelial cells into the bloodstream to establish colonies in the secondary sites. Primary tumours of a mesenchymal nature apparently did not have sufficient plasticity to re-epithelialise at the secondary sites. This is similar to that recently reported for the bladder and prostatic systems described above, where cooperativity between the mesenchymal and epithelial variants for spontaneous metastasis was also demonstrated in the prostatic model both *in vitro* and *in vivo* [42]. However, plasticity of the epithelial variants *in vivo* towards a transient mesenchymal phenotype to facilitate initial invasion away from the primary site was also demonstrated.

The expression of E-cadherin in tumour emboli has been reported in relation to inflammatory breast cancer, a distinct type of invasive breast cancer in which persistent E-cadherin is present in the primary tumour despite its highly aggressive nature [75]. Therefore, E-cadherin expression in local LVI is not altogether surprising; however, seeing E-cadherin-expressing tumour cells in the local lymphovasculature was unexpected as it usually is not seen until the stage of further metastasis in the target organs. These observations support the notion that E-cadherin re-expression facilitates formation of tumour cell emboli by enhancing intercellular adhesion of tumour cells. E-cadherin re-expression leads to altered receptivity towards signals from the extracellular matrix, including growth factors (reviewed in [15]).

6 Influence of microenvironmental factors at the secondary site which may contribute to MET

Lang and colleagues demonstrated that PC3 prostate cancer cells only underwent a MET when plated on three-dimensional Matrigel, as evidenced through the formation of acinar spheroids [76], suggesting a pro-MET influence from the basement membrane substrate and/or from cellular factors expressed within the acinar spheroid microenvironment. The expression of these microenvironmental factors may be determined by the *size* of the metastases, as suggested by Kurahara et al. who demonstrated that larger (greater than 2 mm) lymph node metastases from pancreatic head cancers expressed significantly higher E-cadherin compared to smaller metastases [77]. Interestingly, in micrometastases of prostate cancer to the liver, the inverse was found, where the larger metastases appear to revert back to EMT [78]. Some of these microenvironmental influences may be driven by the cancer cells too, since Korpal et al. [35] showed that miR-200 promotes Sec23A-positive secretory vesicles, the cargo of which may regulate both autocrine and paracrine pathways to promote establishment, survival and/or growth of the macrometastases. The

paracrine pathways may result in recruitment or activation of stromal cell populations.

In the clearest example of MET *in vivo* of breast cancer cells, Chao and colleagues demonstrated E-cadherin expression in lung metastases from E-cadherin-*negative* MDA-MB-231 primary xenografts [23]. They suggested that the re-expression of E-cadherin in metastases was influenced by the microenvironment of the metastatic site. To prove their hypothesis, they demonstrated that the E-cadherin-negative MDA-MB-231 cells express E-cadherin when co-cultured with hepatocytes, a switch they had previously demonstrated in prostate cancer cells cultured under similar conditions [78, 79]. E-cadherin downregulation in cancer cells often occurs as a result of promoter methylation. Taking this into account, they postulated in the MDA-MB-231 study that loss of promoter methylation at the secondary site causes the metastatic cancer cells to re-express E-cadherin through MET. A potential demethylating factor has been identified as 1α,25-dihydroxyvitamin D3, which has been shown to promote *de novo* E-cadherin re-expression in MDA-MB-231 cells [80]. Furthermore, these authors demonstrate that the receptor for this ligand, the vitamin D receptor, is positively expressed in metaplastic carcinomas of the breast.

7 Influence of EMT/MET states on cellular proliferative state

Several lines of evidence suggest that locally invading tumour cells undergoing an EMT proliferate *less* as they migrate *more* [81–84] (summarised in Fig. 4). An early study on well-differentiated colorectal adenocarcinomas with lymph node metastasis has reported loss of the proliferative marker Ki-67 in cells along the invasive front of primary tumours, in contrast to the presence of high Ki-67 at the centre of the tumours. They have observed diminished membranous E-cadherin and nuclear localized β-catenin in these Ki-67-negative cells, suggesting attenuated proliferative capacity in cells that have undergone EMT. Another study by the same group has demonstrated a higher expression of cell cycle inhibitor, $p16^{INK4A}$ (inhibitor of kinase 4), in the invasive front of well-differentiated colorectal carcinomas where β-catenin is localized in the nucleus, when compared to the $p16^{INK4A}$-negative cells with cytoplasmic β-catenin comprising the centre of the tumour, confirming the hindered cell proliferation associated with EMT [84].

A direct causal link between EMT and a downregulation of proliferation may lie with the E-cadherin repressor gene set. For example, Snail1-transfected MDCK cells exhibit an arrest in cell proliferation [85]. Vega and colleagues reported that MDCK cells transfected with the transcription factor Snail underwent a complete EMT and demonstrated abolished cell proliferation resulting from diminished cyclin D1

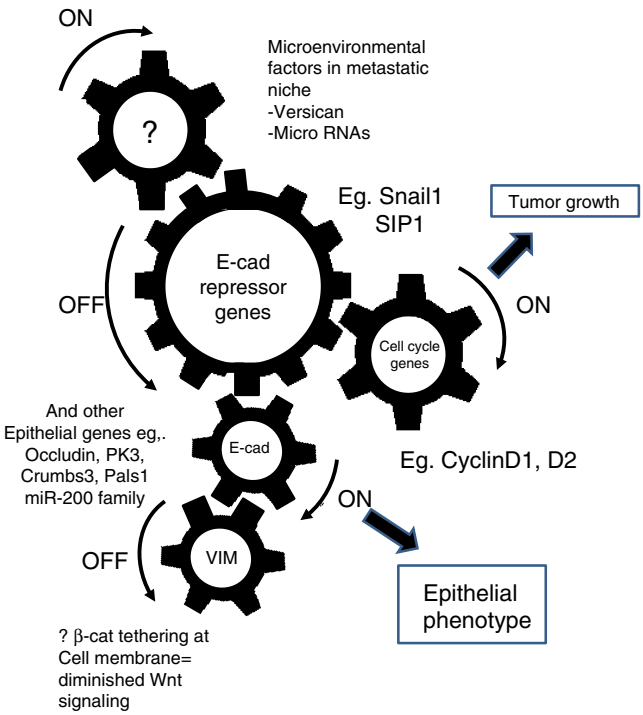

Fig. 4 Schematic depicting the consequence of MET on tumour growth. Mesenchymal cells which have been shed by the primary tumour may end up in the local lymphovasculature, as we observed in the MDA-MB-468 xenograft tumours, or at distant secondary sites. These locations may express factors such as versican, which drives miR-200 expression in the tumour cells to repress E-cadherin repressor genes and hence permit a MET and E-cadherin re-expression to occur. Thus, the driving 'cog' for this phenotypic change may be the expression of these microenvironmental factors, leading to the *repression* of E-cadherin repressor genes (e.g. Snail1/2, Zeb1/2, Twist1/2, etc.) in the tumour cells. In turn, cell cycle-driving genes cyclin D1 and D2, genes that are directly repressed by Snail1 and Zeb2, may be then re-activated, restoring proliferation and tumour growth. An additional consequence of E-cadherin repressor gene repression is the re-expression of other epithelial genes such as occludin and crumbs3, and possibly the re-expression of mesenchymal genes via the tethering of β-catenin by membranous E-cadherin, preventing the activation of the Wnt pathway

and D2 expression. Furthermore, it has been shown that ZEB2-mediated EMT in A431 cells led to the repression of cyclin D1 and inhibition of cell proliferation [86]. Colon cancer cells at the invasive front in which EMT is occurring, coinciding with the region where ZEB1 is expressed, display a downregulation of proliferation [84, 87–89]. Therefore, it can be assumed that EMT can arrest cell proliferation through many EMT regulators such as β-catenin, Snail and ZEBs.

It can therefore be hypothesised that tumours that have undergone an MET at a secondary site become more proliferative. Elegant work by Gao and colleagues have identified bone marrow-derived myeloid progenitor cells as responsible for promoting a favourable premetastatic niche [90]. They identified an essential factor expressed by these cells,

the chondroitin sulphate proteoglycan versican, which promoted a MET in MDA-MB-231 cells. Importantly, this factor also led to an increase in proliferation of this cell line *and* suppression of Snail1. Given the suppressive effect that Snail1 has on the cell cycle as outlined earlier, this may be the mechanism of proliferation re-activation in MDA-MB-231 cells and hence their metastases in the xenograft model, thus providing further insight into the effects of a MET in secondary breast cancers. Therefore, EMT and MET may determine dormant or active states of the tumour, respectively, and allow for an indeterminate number of cycles of invasion and metastases formation.

8 Clinical implications of MET-driven growth of metastases

It has been well documented that cells that have undergone EMT withstand external insults better, leading these cells to display resistance to chemotherapy and radiotherapy [91]. This is particularly evident in non-small cell lung cancer responses to EGFR-targeted therapies, seen both experimentally and in patients [92–94]. Along similar lines, breast cancer cells remaining after neo-adjuvant treatment are enriched for EMT gene expression signatures characteristic of breast cancer stem cells [21, 95]. Indeed, dramatically enhanced EMT and metastasis were demonstrated recently after vascular disruption of mammary tumours using pericyte ablation [96].

Although the exact underlying mechanism is elusive, growing evidence suggests that EMT-associated apoptosis reduction and senescence inhibition contribute largely to therapeutic resistance. Early work has revealed that EMT was responsible for rescuing serum-deprived and TGF-β-treated hepatocytes from apoptosis [97]. It has also been reported that the EMT regulator Snail prevents apoptosis induced by serum deprivation and TNF-α treatment in Madin–Darby canine kidney (MDCK) cells [85]. A recent study has shown the ability of EMT regulators Twist1 and Twist2 to disrupt Ras-induced senescence by inhibiting the p53 and Rb pathways [98]. Arrested cell proliferation and apoptosis have been observed in breast cancer cells that have undergone EMT subsequent to prolonged TGF-β exposure [99]. The EMT regulator Zeb2 has been linked as a preventer of DNA damage-triggered apoptosis in bladder carcinoma cells [100]. With more data accumulating, the association between EMT and reduced apoptosis is becoming more apparent.

It can be hypothesised therefore that tumours that have undergone an MET at a secondary site may be *more* susceptible to apoptotic insults and hence may be treated more successfully with chemotherapeutic drugs. Given also that proliferation may be re-activated in MET, these secondary tumours may also be more amenable to treatment with chemotherapeutic drugs, which act on cell cycle machinery. Unfortunately, this does not translate into clinical efficacy of our current chemotherapies, and this is presumed to be due to the bulk of the established metastases, which are not amenable to surgical resection/debulking the way many primary tumours are. More progress is needed to combat these larger metastases, and understanding the EMP axis may ultimately prove useful.

On the other hand, can subclinical tumour be forced to undergo a MET to facilitate therapy? This suggestion has been made, since translated to the clinic, this could re-awaken the dormant, clinically silent tumour cells and render them chemoresponsive. Along these lines, clinical trials in the 1980s and early 1990s were designed to re-awaken indolent tumour deposits with growth factors to drive proliferation prior to radiation and/or chemotherapy, as had shown promise in preclinical mouse models. However, these approaches did not prove useful in human tumours and thus were stopped. More work is needed in this area to strategise around the possibilities of manipulating EMP in conjunction with chemotherapy.

An emerging possibility is that after MET, the micrometastatic tumour cells can establish cell heterotypic interactions via E-cadherin binding, as recently described [101]. Such E-cadherin attachments are considered to initiate contact inhibition and suppression of proliferation (thus, the designation of E-cadherin as a tumour suppressor). As our current cancer armamentarium targets by and large only rapidly proliferating cells, this reduction in cycling would be noted as chemoresistance of this small, cryptic nodules [34].

In sum, there is ample biological precedence for viewing the MET in the metastatic site as promoting either chemosensitivity or chemoresistance. Thus, experimental model systems will be needed to settle this key question as it directly impinges on whether inducing or inhibiting MET would be beneficial in the treatment of breast cancer. Further, the question of whether the MET is stable in the metastases or if these cells show ongoing phenotypic plasticity leading to a second EMT is also open to question. What can be said is that the view of tumour progression as a phenotypically plastic continuum rather than a relentless regression towards greater and greater degrees of dedifferentiation has opened numerous novel avenues with which to explore the biology and medicine of breast cancer metastasis.

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